Plant Molecular Biology **18**: 1083–1089, 1992.

The activation process of *Arabidopsis thaliana* A1 gene encoding the translation elongation factor EF-1α is conserved among angiosperms

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Received 28 June 1991; accepted in revised form 7 January 1992

Key words: translation elongation factor genes, promoter analysis, evolution

Abstract

In *Arabidopsis thaliana*, the activation process of the A1 EF-1α gene depends on several elements. Using the GUS reporter gene, transient expression experiments have shown that mutations of upstream *cis*-acting elements of the A1 promoter, or the deletion of an intron located within the 5′ non-coding region, similarly affect expression in dicot or monocot protoplasts. The results reported here strongly suggest that this 5′ intron is properly spliced in *Zea mays*. We show that two *trans*-acting factors, specifically interacting with an upstream activating sequence (the TEF 1 box), are present in nuclear extracts prepared from *A. thaliana*, *Brassica rapa*, *Nicotiana tabacum* and *Z. mays*. In addition, a DNA sequence homologous to the TEF 1 box, found at approximately the same location within a *Lycopersicon esculentum* EF-1α promoter, interacts with the same *trans*-acting factors. Homologies found between the *A. thaliana* and *L. esculentum* TEF 1 box sequences have allowed us to define mutations of this upstream element which affect the interaction with the corresponding *trans*-acting factors. These results support the notion that the activation processes of *A. thaliana* EF-1α genes have been conserved among angiosperms and provide interesting data on the functional structure of the TEF 1 box.

Introduction

The elongation factor EF-1α belongs to the housekeeping protein family and has been intensively studied because of its central role in the protein synthesis process [23]. In spite of these studies, except for *Saccharomyces cerevisiae* [15] and *Arabidopsis thaliana* [8], there are no published data concerning the characterization of *cis*- and *trans*-acting elements involved in the activation of EF-1α genes.

In *A. thaliana*, EF-1α is encoded by a small multigenic family of four members (A1-A4) which are all actively transcribed [1, 19]. Using *Arabidopsis* protoplasts, transfection experiments have shown that the A1 promoter mediates a transient expression higher than that obtained using the cauliflower mosaic virus (CaMV) 35S promoter, one of the strongest promoters known both in transient expression systems and stably transformed plant cells [1]. This high level of expression appears to be conserved in transgenic

Arabidopsis (unpublished data). Deletion analysis of A1 promoter has shown that several elements are involved in the transcription activation process in transfected *Arabidopsis* cells [8]. The location of these elements within the A1 promoter is indicated in Fig. 1A. A DNA sequence located upstream of position −289 relative to the transcription initiation site is required for a maximum activity. One *cis*-acting domain, the TEF 1 box, has been accurately mapped 100 bp upstream of transcription initiation site. This domain is the target for *trans*-acting factors identified in nuclear extracts prepared from *Arabidopsis* leaves or cell suspension cultures. In addition, we have shown that the A1 gene contains an intron, located within the 5′ non-coding region. Evidence has been obtained that this intron is essential to the expression of the GUS reporter gene used in the transient expression experiments. [8].

Here, we report results showing that mutations of the A1 promoter similarly affect the expression in dicot or monocot protoplasts and that factors interacting with the TEF 1 box are present in nuclear extracts prepared from *A. thaliana*, *Brassica rapa*, *Nicotiana tabacum* and *Zea mays*. Moreover, we show that a related DNA sequence found at approximately the same location within the Le EF-1 *Lycopersicon esculentum* EF-1α promoter [25], specifically interacts with the same *trans*-acting factors. These data have allowed us to define by mutagenesis, two important domains within the TEF 1 box.

Material and methods

Transient gene expression assays

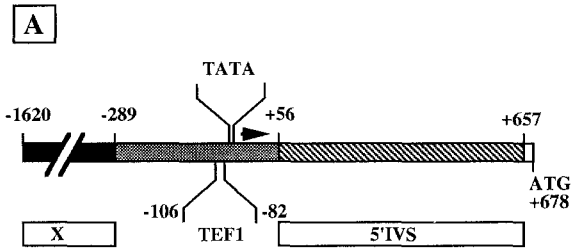
The plasmid constructions used to transfect the protoplasts have been already described [8]. The protoplasts were prepared from cell suspension cultures of *A. thaliana* [1] and of *Z. mays* (49S1 embryogenic cell line developed by T. Hardy, Rhône Poulenc Agro), or from *N. tabacum* leaves [7]. For *A. thaliana* and *N. tabacum*, transfections were performed according to the $Ca(NO_3)_2$ – PEG procedure [22]. The electroporation method was used for the transfection of *Z. mays* protoplasts [9]. In all cases, 20 μg of plasmid DNA, carrying the indicated promoter configuration fused to the GUS reporter gene, were used to transfect 10^6 protoplasts. The GUS activity was measured after 20 h by fluorometry [16].

Gel retardation assays

Crude nuclear extracts were prepared from young leaves of *B. rapa*, *N. tabacum* or *Z. mays*, using the procedure described for the preparation of *A. thaliana* nuclear extracts [8]. Binding assays were done in a volume of 30 μl containing 10 mM TRIS-HCl pH 8, 50 mM NaCl, 7 mM 2-mercaptoethanol, 10% glycerol, 2 μg of poly (dI-dC), 1000–5000 cpm (0.1–0.5 ng) of ^{32}P-labelled probe and 2–5 μg of nuclear proteins. After 20 min at 25 °C, the free and bound DNA were separated on 5% polyacrylamide gels in 0.5× TBE.

Results

In order to determine whether the activity of *cis* elements involved in the activation of the *A. thaliana* EF-1α A1 promoter was conserved among angiosperms, characteristic promoter mutations were tested in *N. tabacum* and *Z. mays* protoplasts. Figure 1A shows a schematic representation of the A1 promoter, with the location of the relevant *cis* elements [8]. The GUS activities given in Fig. 1B are expressed relative to the full-length promoter (x-TEF-TATA-IVS configuration: 2300 bp upstream of the AUG translation initiation codon fused to the GUS reporter gene). As in *A. thaliana*, in transfected *Z. mays* protoplasts the full-length A1 promoter appears to drive a GUS activity higher than that obtained using the CaMV 35S promoter (CaMV 35S configuration). A 5′ deletion down to the position −111 relative to the transcription initiation site (TEF-TATA-IVS configuration), led to about a two-fold decrease in the GUS activity. This moderate effect, which depends on a *cis*-acting element



B

Promoter configuration	*A. thaliana*	*N. tabacum*	*Z. mays*
X-TEF-TATA-IVS	100	100	100
TEF-TATA-IVS	40+/-5	60+/-2	51+/-8
TATA-IVS	2,5+/-0,2	4+/-1	3+/-2
TEF-TATA	3+/-0,2	ND	2+/-1
CaMV 35S	42+/-5	ND	21+/-4

Fig. 1. A. Schematic representation showing the location of EF-1α A1 promoter elements involved in the activation of expression [8]. The positions are relative to the transcription initiation site. See the text for details. B. Transient expression in *A. thaliana*, *N. tabacum* and *Z. mays* protoplasts. The nature of the promoter configuration indicated is detailed in the text. The relative GUS activities, normalized to the full length promoter (100%), are the mean of data from at least three independent transfections. After 20 h, the GUS activity measured for the full length promoter was 120, 6 or 30 nmol/min per mg protein for *A. thaliana*, *N. tabacum* or *Z. mays* protoplasts respectively. ND, not determined.

located upstream of the position −289, appears to be conserved among the three species tested (two dicots and one monocot). Deletion down to the position −100 (TATA-IVS configuration), produced a dramatic drop of GUS activity in *A. thaliana* as in *N. tabacum* and *Z. mays*. This deletion disrupts an upstream activating sequence (the TEF1 box; see Fig. 1A and 4A) required for expression in *Arabidopsis*. Therefore, our results suggested that the function of the TEF 1 box was conserved in *N. tabacum* and *Z. mays*. To confirm this point, we undertook a search for protein-DNA interactions in the region of the TEF 1 box, using nuclear extracts prepared from various plants. Using *Arabidopsis* nuclear extracts, we have previously shown that two complexes (C1 and C2) are formed with a DNA probe extending from position −111 to +46 relative to the

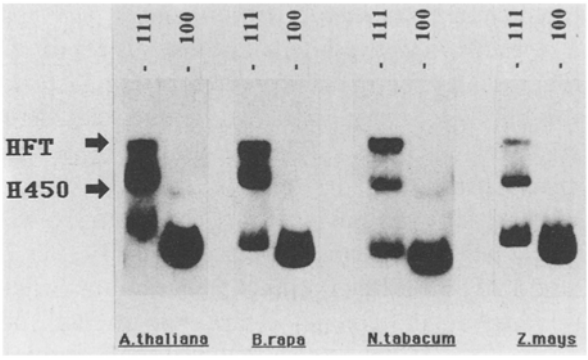


Fig. 2. Identification by gel retardation of *trans*-acting factors interacting with the TEF1 box. The probes used extend from the 5′ position −100 or −111 to the position +46 relative to the transcription initiation site. The origin of nuclear extracts used is indicated. HFT and H450 correspond to C1 and C2 complexes previously described [8] (see the text for details).

transcription initiation site and that these two complexes disappear using a probe deleted down to position −100 [8]. Therefore, the same DNA fragments (−111/+46 and −100/+46) were used as probes to test for the presence of *trans*-acting factors specifically interacting with the TEF 1

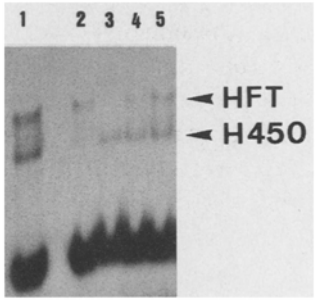


Fig. 3. Fractionation of HFT and H450 on Heparin-Ultrogel. A crude nuclear extract prepared from *Z. mays* was chromatographed on Heparin-Ultrogel. The crude extract was loaded on the column at an ionic strength of 50 mM NaCl in the nuclear extraction buffer [8]. The elution was done step by step by increasing of 100 mM NaCl the ionic strength at each step. The binding activity was tested for each fraction by gel retardation assays, using as probe a 50-mer double-stranded oligonucleotide corresponding to a sequence overlapping the *Arabidopsis* TEF 1 box (sequence underlined in Fig. 4A). 1, crude nuclear extract; 2, flow-through fraction; 3, 450 mM NaCl fraction; 4 and 5, as in 3 but supplemented by half (4) or the same amount (5) of flow-through fraction proteins used in 2.

box, in nuclear extracts prepared from *A. thaliana*, *B. rapa*, *N. tabacum* or *Z. mays*. The results of gel retardation experiments reported in Fig. 2 show that, as in *Arabidopsis*, two complexes were invariably observed with the −111 probe, but absent using the −100 probe. These two DNA binding activities have distinct chromatographic properties on heparine-Sepharose (Fig. 3, lanes 2 and 3). Under the experimental conditions used (loading on the column at an ionic strength of 50 mM NaCl), the protein involved in the formation of the upper complex (C1) is recovered in the flow-through fraction whereas that involved in the formation of the lower complex (C2) is eluted from the column at an ionic strength of 450 mM NaCl. For this reason, the C1 and C2 complexes [8] have been renamed HFT (for heparin flow through fraction) and H450 (for heparin 450 mM NaCl fraction) respectively. In binding assays, the mixture of HFT and H450 does not reveal cooperative effects in their DNA binding properties (Fig. 3, lanes 4 and 5).

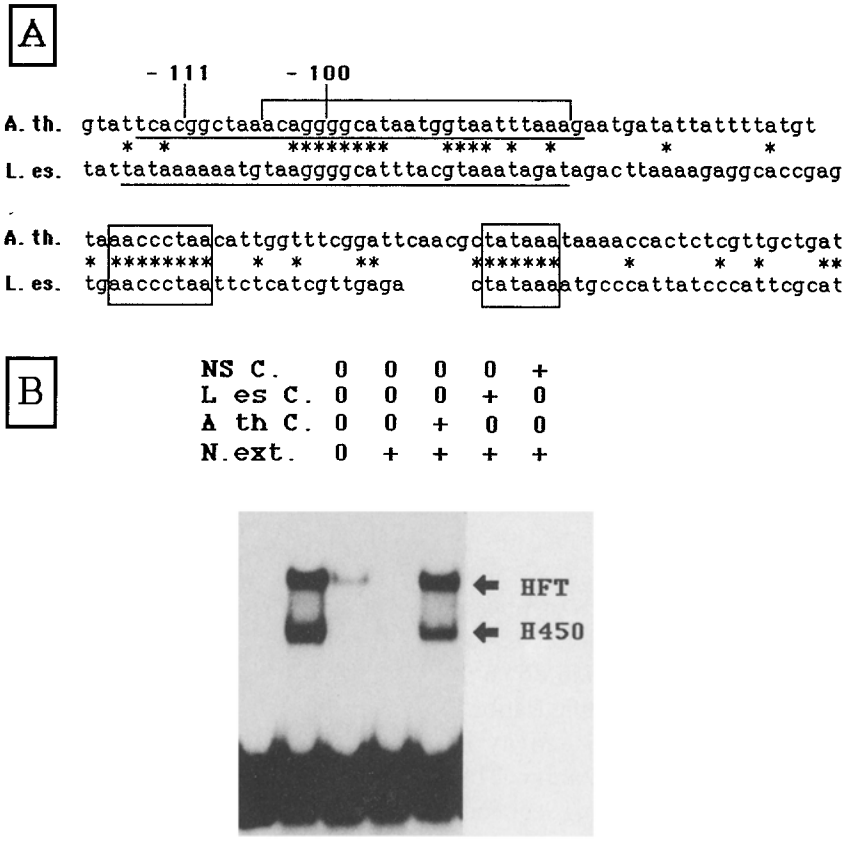


Fig. 4. A. Comparison between the sequences located upstream of the cap site of the EF-1α *A. thaliana* A1 gene (A. th.) [1] and the corresponding region of the *L. esculentum* Le gene (L. es.) [25]. Similarities are indicated by asterisks between the sequences. The TATA and the Telo motifs are boxed. The square bracket above the A1 sequence represents the protected region of HFT and H450 complexes against OP-Cu cleavage [8]. The *A. thaliana* or *L. esculentum* underlined sequences correspond to double-stranded synthetic oligonucleotides used for the gel retardation experiments reported in B. The indicated −111 and −100 positions are relative to the transcription initiation site of *A. thaliana* A1 gene. B. Gel retardation experiment showing the conservation of the TEF-1 element in *L. esculentum*. A double-stranded synthetic oligonucleotide corresponding to the *L. esculentum* sequence underlined in A was used as probe. When indicated, the binding reaction was supplemented with 10 ng of competitor (molar excess of competitor: 50). NS C., non-specific competitor corresponding to the double-stranded oligonucleotide 5′-TCGAATTCTATGTTAAACCCTAACATTGATATCGA-3′. L es C. and A th C., *L. esculentum* and *A. thaliana* competitors respectively, corresponding to the sequences underlined in A. N. ext., 2 μg of *Z. mays* nuclear extract. HFT and H450 correspond to C1 and C2 complexes previously described [8] (see text for details).

In plants, in addition to the four *A. thaliana* genes encoding EF-1α, the structure of a *L. esculentum* EF-1α genomic clone has been recently reported [25]. Figure 4A shows an alignment between sequences located immediately upstream of the cap site of the *A. thaliana* EF-1α A1 gene and the corresponding region of the *L. esculentum* EF-1α gene. Upstream of a putative TATA box of the *L. esculentum* promoter, two blocks of homologies were found with the *Arabidopsis* A1 promoter. The first one concerns a strikingly conserved sequence, the telo box, homologous to the repeat motif of higher eukaryote telomeres and found at the same location within the four *A. thaliana* EF-1α promoters [1]. The second one is homologous to the TEF 1 box. This observation prompted us to test the ability of the *L. esculentum* TEF 1-box like sequence to interact with the HFT and H450 proteins. Figure 4B shows the results of a gel retardation experiment conducted using *Z. mays* nuclear extracts and a labelled probe consisting of a double-stranded synthetic oligonucleotide containing the TEF 1-like sequence of *L. esculentum*. This synthetic probe was able to form retarded complexes which were competed by *A. thaliana* or *L. esculentum* TEF 1 unlabelled sequences. In contrast, an unrelated double-stranded oligonucleotide corresponding to A1 promoter sequences extending from positions −70 to −45 relative to the transcription initiation site (see Fig. 4a), did not prevent the formation of these complexes. These data clearly show that the HFT and H450 proteins specifically interact with the putative TEF 1 box of *L. esculentum*. Moreover, they suggest that the sequence AGGGGCATNNNNGTAANTNNA (Fig. 4A) is a basic motif required for the recognition of the TEF 1 box by these proteins. In order to confirm this assumption, two mutated TEF 1 boxes derived from the *Arabidopsis* TEF 1 box (Fig. 4A, upper line), have been used as probe in binding assays using *A. thaliana* nuclear extracts. In the first mutation (mt 1 probe), within a 50-mer double-stranded oligonucleotide corresponding to sequences overlapping the TEF 1 box (wt probe), the sequence ...AGGGGCAT... has been replaced with ...AGAAACAT... In the second one (mt 2 probe), the sequence ...GGTAA... has been replaced with ...GAGCA... The results of gel retardation experiments reported in figure 5 show that the mt 1 mutation, as the mt 2 mutation, affect the interaction of HFT and H450 proteins with the TEF 1 box, showing the functional importance of conserved sequences AGGGGCAT and GTAA within the TEF 1 box.

In addition to the two upstream elements mentioned above (the *x* element and the TEF-1 box), we have already shown that an intron located within the 5′ non-coding region of A1 gene was essential to obtain a maximum expression in *A. thaliana* protoplasts [8]. The results reported in Fig. 1B show that the role of this 5′ intron in the activation of the expression is conserved in a monocot. A chimaeric construct, obtained by substitution of genomic sequences located upstream of the AUG translation initiation codon by their corresponding cDNA sequences (TEF-TATA configuration), led to a 20-fold lower GUS activity in *Z. mays* protoplasts compared to that obtained using the TEF-TATA-IVS promoter configuration.

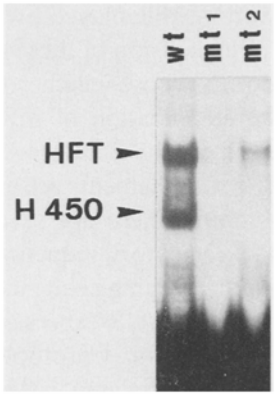


Fig. 5. Gel retardation assays showing the effect of TEF 1 box mutations on the formation of HFT and H450 complexes. *wt probe*: synthetic double-stranded oligonucleotide probe, 5′-gggtctagaGGCTAAACAGGGGCATAATGGTAATTTAAAGAATcgatggg-3′, corresponding to the *Arabidopsis* DNA sequence overlapping the TEF 1 box (see Fig. 4A); *mt 1 probe*: 5′-gggtctagaGGCTAAACAGaaaCATAATGGTAATTTAAAGAATcgatggg-3′; *mt 2 probe*: 5′-gggtctagaGGCTAAACAGGGGCATAATGgtaATTTAAAGAATcgatggg-3′.

Discussion

In the majority of cases where genes have been transferred into heterologous plants, qualitative aspects of expression are maintained. However, this conservation appears to depend on the evolutionary distance between donor and host plants. Thus, monocot-dicot transfers often result in no or inappropriate expression of the transferred gene [see 28 and references therein]. The data reported in this paper show that *cis*- and *trans*-acting elements involved in the transcription activation of the *Arabidopsis* EF-1α A1 gene are conserved among angiosperms.

The results obtained in *Z. mays* are interesting in several respects. They show that in a monocot, the A1 promoter is about four fold more efficient than the CaMV 35S promoter and that this expression depends on the same *cis*-acting elements as those required for expression in *A. thaliana*. As in *Arabidopsis*, the intron which interrupts the 5′ untranslated A1 RNA leader is essential to obtain an optimum GUS activity. It must be noted that such 5′ IVS have been found in all the higher eukaryotic EF-1α genes characterized up to now [14, 18, 25, 27]. The conservation during the evolution of this structural organization probably reflects an important role played by these 5′ IVS in the control of expression of these genes. At the moment, we do not know whether this intron is involved in the stabilization of mRNA precursors, as suggested for the *Z. mays* Adh1 intron 1 [5], whether *cis*-acting elements are present within the 5′ IVS, or both. There are several cases in animals in which regulatory sequences have been localized within intervening sequences [e.g. 4, 6, 24]. The high level of GUS expression driven by the A1 promoter in *Z. mays* protoplasts strongly suggests that the intron located within the 5′ non-coding region is properly spliced. Several AUG codons, in frame with the GUS reporter gene, are present within this intron. All of them are followed by stop codons, excluding that in *Z. mays*, alternative translation initiations could occur from these AUG codons on mRNA precursors. These data contrast with the assumption that the fidelity with which processing signals of heterologous RNA are recognized in monocots and dicots can vary considerably [17]. However, it must be noted that this assumption results from experiments where monocot genes have been transferred in dicots. From considerations of the much higher G + C content of introns in monocots than dicots, and the possible consequences of this base composition on the secondary structures of mRNA precursors, it has been suggested and shown that most of the primary transcripts from dicot genes could be efficiently spliced in monocots, but not the contrary [3, 11].

In all plants analysed, we have shown that at least two nuclear factors interacted with the *A. thaliana* TEF 1 box and that the same factors bound a putative TEF 1 box from a *L. esculentum* EF-1α promoter. Many plant DNA binding proteins which interact with promoter elements of diverse genes have now been characterized [see 13 and references therein]. The comparison between the consensus target sequences for these DNA-binding proteins and the TEF 1 box does not reveal striking homologies. The sequence AGGGGCAT found within the 5′ part of the TEF 1 box of *A. thaliana* and *L. esculentum* shares some resemblance to the evolutionarily conserved G box ACGTGGCAPy recognized by the nuclear factor GBF [10]. Similarly, the conserved GTAA motif of the 3′ part of the TEF 1 box is also found within the ocs element of T-DNA and plant viral promoters and within the box II* of pea rbcS promoters [2, 12, 26].

The presence of the two DNA binding activities HFT and H450 in all the plants examined so far, together with their distinct chromatographic properties, suggest the presence of two different proteins which could combine their effect in an exclusive manner by interacting with overlapping sequences. Such an exclusion mechanism has been already described in yeast and in animals (e.g. 20, 21]. The two TEF 1 box mutations tested in this work, similarly affect the interaction with the HFT and H450 proteins (Fig. 5), suggesting that the sequences required for the formation of HFT and H450 complexes are identical or overlap. A collection of TEF 1 box point mutations is required to test whether the overlapping se-

quences involved in the formation of HFT and H450 complexes are identical or not, and to study the role of each one of these two complexes in the activation or in the repression of the expression process of EF-1α genes.

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