Mol Genet Genomics (2001) 265: 763–770
DOI 10.1007/s004380100478

ORIGINAL PAPER

K. Wu · K. Malik · L. Tian · M. Hu · T. Martin
E. Foster · D. Brown · B. Miki

Enhancers and core promoter elements are essential for the activity of a cryptic gene activation sequence from tobacco, tCUP

Received: 15 December 2000 / Accepted: 25 February 2001 / Published online: 31 March 2001

Abstract Cryptic gene regulatory elements are sequences that are inactive at their native locations in the genome but have the ability to become functional when positioned adjacent to genes. We have recently isolated such a cryptic sequence from tobacco, tCUP, that can act as a promoter. A 135-bp fragment spanning extending from position –197 to –62, relative to the transcription start site, was found to promote GUS expression in all of the major organs of transgenic *Arabidopsis* plants. Furthermore, this 135-bp fragment complemented the –46 minimal promoter of CaMV 35S and conferred constitutive expression on transgenic *Arabidopsis* plants. An electrophoretic mobility-shift assay showed that nuclear proteins prepared from tobacco leaves interact with the 135-bp fragment. tCUP has a core promoter that lacks the TATA consensus sequence but addition of a TATA-box sequence increased the core promoter activity by three-fold. The sequence surrounding the transcription start site of tCUP has sequence similarity with the initiator element (Inr), and deletion of this sequence significantly reduced promoter activity, suggesting that an essential Inr element may exist in the tCUP core promoter. Fusion of the GCC-box enhancer element from pathogenesis-related genes to the core promoter elevated tCUP core promoter activity. Our study indicates that cryptic promoters are similar in composition and organization to promoters associated with expressed genes and that their promoter elements can be combined to create composite promoters that are fully functional. This data provides direct evidence that the expression pattern of plant genes can be influenced by cryptic gene regulatory elements when they are brought into juxtaposition with genes through DNA rearrangements.

Keywords Cryptic promoter · tCUP · Enhancers · Core promoter · Transgenic plant

Communicated by J. Schell

K. Wu · K. Malik · M. Hu · T. Martin · E. Foster · B. Miki (✉)
Eastern Cereal and Oilseed Research Centre,
Agriculture and Agri-Food Canada, Ottawa,
Ontario, Canada K1A 0C6
E-mail: mikib@em.agr.ca
Tel.: +1-613-7591685
Fax: +1-613-7591701

L. Tian · D. Brown
Southern Crop Protection and Food Research Centre,
Agriculture and Agri-Food Canada, London,
Ontario, Canada N5V 4T3

B. Miki
Agriculture and Agri-Food Canada,
Room 2091 K.W. Neatby Building, Ottawa,
Ontario, Canada K1A 0C6

Introduction

The use of promoter-trap vectors with marker genes that lack promoters or possess a minimal 35S promoter has led to the discovery of a variety of plant promoter activities (Koncz et al. 1989; Fobert et al. 1991; Goldsbrough and Bevan 1991; Topping et al. 1991; Lindsey et al. 1993; Sundaresan et al. 1995; Cocherel et al. 1996). It has been generally assumed that activation of such promoterless or enhancerless marker genes results from insertion within or immediately adjacent to expressed genes (Koncz et al. 1989; Topping et al. 1994; Wei et al. 1997). However, further investigations are showing that integration may also activate silent regulatory sequences that are not associated with genes (Lindsey et al. 1993; Fobert et al. 1994; Ökrésza et al. 1998; Foster et al. 1999; Plesch et al. 2000). Inactive regulatory sequences with the ability to become functional when positioned adjacent to genes have been found in many different organisms and are referred to as "cryptic" elements (Al-Shawi et al. 1991; Fourel et al. 1992; Irniger et al. 1992; Fobert et al. 1994; Troyanovsky and Leube 1994; Tee et al. 1995). The isolation and characterization of several cryptic promoters from plants (Fobert et al. 1994; Ökrésza et al. 1998; Foster et al. 1999; Plesch et al. 2000) suggests that cryptic gene regulatory elements might be abundant in the plant

genome. It is known that the frequent rearrangements, insertions and deletions that occur in intergenic regions can have a profound effect on gene expression patterns in plants by altering gene promoters (Chen et al. 1997), and it has been suggested that such events may be pivotal to the evolution of higher organisms (Doebley and Lukens 1998). It is therefore conceivable that recruitment from the reservoir of cryptic regulatory elements in the genome may also play a vital role in the survival and adaptation of the higher plants. If this hypothesis is true, we should expect to find examples of cryptic gene regulatory elements that are similar to those associated with expressed genes, and these elements should be able to complement each other in composite promoters. To date this expectation has not been verified.

A typical eukaryotic promoter associated with an expressed gene contains a core promoter region and upstream promoter regions (Singh 1998). The core promoter comprises the transcription start site and flanking sequence that interact with the general transcription machinery; whereas the upstream promoter regions serve as the binding sites for gene-specific regulators. Most core promoters contain a TATA box located ~30 nucleotides upstream of the transcription start site, which directs the preinitiation complex to the promoter through the action of TATA-binding proteins. In the absence of a TATA box, an initiator element (Inr) can compensate by binding the preinitiation complex to the transcription start site (Javahery et al. 1994; Smale 1997; Weis and Reinberg 1997). We are interested in determining whether cryptic promoters have the same modular organization as promoters associated with expressed genes. Knowledge of the specific elements essential for the activity of cryptic promoters will enhance our understanding of transcriptional regulation and its evolution in plants.

tCUP is a constitutive cryptic promoter isolated from tobacco (Foster et al. 1999). Here, we analyzed the components and organization of the tCUP promoter by testing the promoter activity of a series of truncated tCUP fragments in transgenic tobacco. We also replaced the promoter elements with those of the CaMV 35S promoter and analyzed the activity. Our study indicates that the cryptic promoter tCUP contains a core promoter and upstream promoter regions, like promoters associated with expressed genes, and that these elements can be exchanged to create functional modular promoters. To our knowledge, this is the first study on the composition and organization of cryptic promoter elements in plants.

Materials and methods

Plasmid construction

Promoter deletion and replacement constructs were created in the vector pBI221 (Clontech), which contains the GUS (*uidA*) coding region driven by the 800-bp fragment of the CaMV 35S promoter and the *NOS* terminator. Independent promoter constructs representing 5′ promoter deletions of the tCUP were generated at convenient restriction sites within the tCUP sequence (GenBank Accession No. AF133844). The CaMV 35S promoter of pBI221 was replaced with the deletion fragments of tCUP to generate –1639-GUS, –1304-GUS, –394-GUS, –197-GUS and –62-GUS. The numbers represent the distance in bp from the transcription initiation site (Foster et al. 1999).

The promoter fragment used to test the enhancer elements present between positions –197 and –62 of the tCUP (Foster et al. 1999) was amplified with *Taq* DNA polymerase using the primer pair pr-1 and pr-2 (Table 1). All primers had additional nucleotides at the 5′ ends to provide the *Pst*I or *Nsi*I restriction sites for subcloning PCR products. The PCR products were ligated into the *Pst*I site located upstream of the –197-GUS to generate the –197(2X)-GUS construct.

A –46 minimal 35S promoter (–46-35S) was generated by PCR using the primers pr-3 and pr-4 (Table 1) and pBI221 DNA as a template. The PCR product was digested with *Pst*I and *BamH*I, and the resulting fragment was used to replace the *Pst*I-*Bam*HI fragment in pBI221. The –197 to –62 fragment of tCUP was subcloned into the *Pst*I site located upstream of the –46-35S-GUS to generate the constructs –197-35S-GUS, –197R-35S-GUS and –197(2X)-35S-GUS.

The –12-GUS construct was generated by PCR using pr-5 and pr-4 as primers. The PCR product was digested with *Xba*I and *Kpn*I, and the resulting fragment was used to replace the *Xba*I-*Kpn*I fragment in tCUP-GUS. To generate the –62-tsr-GUS construct, the DNA sequence between –62 and –12 of tCUP was amplified with the primers pr-6 and pr-7. The PCR product was digested with *Xba*I and *Nde*I, and the resulting fragment was used to replace the *Xba*I-*Nde*I fragment in tCUP-GUS. The TA30-GUS construct was generated using pr-8 and pr-4. To generate the GCC-62-GUS construct, a 51-bp fragment (GCATAAGAGCCGCCACTAAAATAAGACCGATCAAATAAGAGCCGCCATGCA) containing two GCC boxes (underlined; Ohme-Takagi and Shinshi 1995) was ligated into the *Pst*I site located upstream of the –62-GUS construct.

DNA sequence analysis

All PCR products were sequenced to make sure that no mutations had been introduced into the sequence. Dye primer sequencing of plasmid DNA was performed using an automated sequencing system (Applied Biosystems). DNA sequence analysis was carried out using the DNASIS program (Hitachi Software Engineering).

Plant transformation and particle gun delivery assays

Tobacco and transgenic *Arabidopsis thaliana* plants were generated as described previously (Foster et al. 1999; Wu et al. 2000). Between 15 and 30 independent transgenic lines for each construct were selected and used for the analysis of GUS activity. Particle Gun

Table 1 Oligonucleotides used in PCR to create constructs

Primer[a]	Sequence (5′→3′)
pr-1S	GGAGCTGCAGGCTATTTAAATACTAGCC
pr-2A	TCAAATGCATGGATCAAAAGGGGAAAC
pr-3S	CACTCTGCAGGCAAGACCCTTCCTCTATA
pr-4A	ATATAAGCTTTGGGGTTTCTACAGGACG
pr-5S	GAGAAGATCTCCAAACACCCCTAACTCTATC
pr-6S	TTGATCATTTTGATCAACGCC CAG
pr-7A	AGGGGGTGCATATGAATTAAAAAAGGAAAAG
pr-8S	ATCATCTAGATCAACGCCCAGAATTTCC-CTATATAATTTTTTAATTCCCAAACAC[b]

[a]S and A indicate sense and antisense primers, respectively
[b]The TATA consensus sequence introduced by this primer is underlined

Delivery Assays were performed as described previously (Wu et al. 2000).

GUS assays

To assay GUS activity, leaves were harvested and frozen in liquid nitrogen. Frozen leaf tissue (100–200 mg) was ground in extraction buffer consisting of 50 mM sodium phosphate pH 7.0, 10 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 0.1% Triton X-100, 10 mM 2-mercaptoethanol and 0.1% sodium lauryl sarcosine. The extract was sonicated twice for 20 s each in a bath-type sonicator and centrifuged for 15 min (4°C), then the supernatant was used for assays of GUS.

For fluorimetric GUS assays (Jefferson 1987), 50 µl of the crude extract from leaf tissues was incubated at 37°C in the presence of 1 mM 4-methylumbelliferyl glucuronide in 0.3 ml of GUS assay buffer [50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% (v/v)Triton X-100, 10 mM β-mercaptoethanol]. After 0, 0.5, 1 and 2 h of incubation, 0.1-ml aliquots were removed and added to 1.9 ml of 0.2 M Na_2CO_3 to terminate the reaction. GUS activity was expressed as pmol of 4-methylumbelliferone produced per mg of protein per min.

For histochemical GUS assays, tissue was incubated in a 0.5 mg/ml solution of 5-bromo-4-chloro-indolyl β-D-glucuronide in 100 mM sodium phosphate buffer pH 7.0, infiltrated in a vacuum for 30 min and incubated at 37°C overnight. Following the incubation, tissue was washed in 70% ethanol to remove the chlorophyll.

Electrophoretic mobility-shift assay

Young tobacco leave were harvested and frozen in liquid nitrogen. Nuclear extract was prepared essentially according to the method described by Green et al. (1988). The tCUP promoter region from –197 to –62 was amplified by PCR. The PCR product was digested with *Pst*I and *Nsi*I, and the resulting fragment was purified from an agarose gel and end-labeled with [γ^{32}P]dCTP using polynucleotide kinase.

The DNA-protein binding reaction was performed in 10 µl of solution containing 0.5 ng of labeled probe, 10 µg of nuclear protein, 2.5 µg of poly(dI-dC).poly(dI-dC), 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl and 50 mM TRIS-HCl, pH 7.5. After a 30-min incubation at room temperature, the samples were loaded onto a 5% polyacrylamide gel and electrophoresed at 4°C for 2 h at 150 V. The gels were dried in a gel dryer and labeled bands were localized by autoradiography.

Results

Deletion analysis of the tCUP promoter

To determine if tCUP is modular and to delineate functional regions of the tCUP promoter, several 5′ deletion constructs were made (Fig. 1A), and their promoter activities were examined in leaves of transgenic tobacco plants. As shown in Fig. 1B, all sequences from –2054 to –394 relative to the transcription initiation site of the tCUP promoter could be deleted with no significant effect on promoter activity. Deletion of sequences to –197 decreased expression by about 60%, compared with the full-length tCUP promoter. The –62 deletion construct greatly reduced GUS activity to a level that was only slightly above background. These results indicated that enhancers or positive *cis*-regulatory elements were located in the regions from –394 to –197 and –197 to –62.

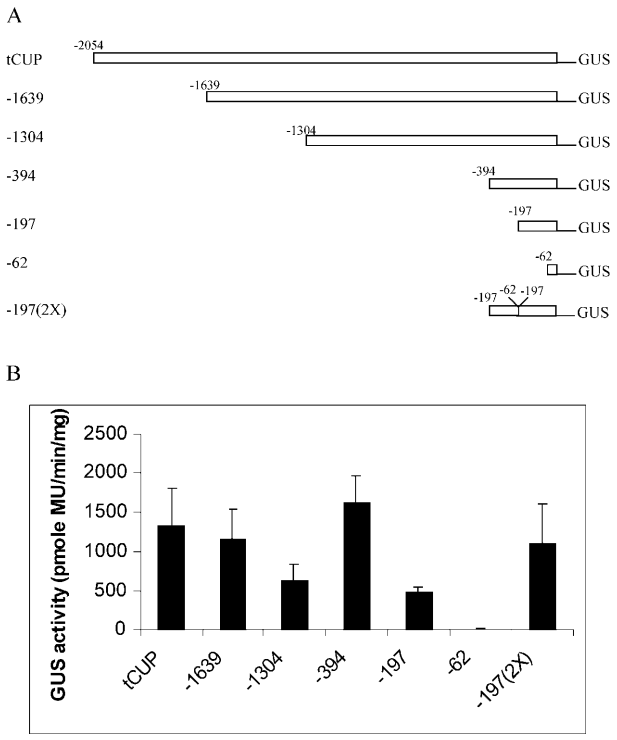

Fig. 1A, B Effect of 5′ deletions on tCUP promoter activity. **A** Schematic diagram of the 5′ deletion chimerical constructs. –197(2X)-GUS is the construct used to test the effect of a duplication of the tCUP upstream region (–197 to –62) on promoter activity. The *numbers above the bars* indicate deletion end points relative to the transcription initiation site (+1) of the tCUP promoter. **B** Average GUS specific activity (pmol MU/min/mg protein) detected in transgenic tobacco plants containing the constructs depicted in **A**. In all, 15–30 independent transgenic plants were tested for each construct. The error bars indicate the standard error of the mean

To reveal enhancer activity within the –197 to –62 segment, this fragment was duplicated in the promoter construct –197(2X)-GUS (Fig. 1A), and GUS activity was analyzed in transgenic tobacco plants. As shown in Fig. 1B, duplication of the –197 to –62 fragment increased promoter activity about two-fold, when compared with the constructs with only one copy of this fragment.

Analysis of the core promoter region

To analyze the tCUP core promoter region, deletions or modifications were made in the segment surrounding the transcriptional start site (Fig. 2A). Promoter activities were examined using a transient expression assay in tobacco leaves (Fig. 2B). Deletion of the –62 core promoter to –12 did not affect GUS activity. Deletion of the sequence surrounding the transcription start site reduced the activity to 40% of that of the –62-GUS construct, suggesting that this sequence was essential for tCUP promoter activity. Replacement of the sequence –30 to

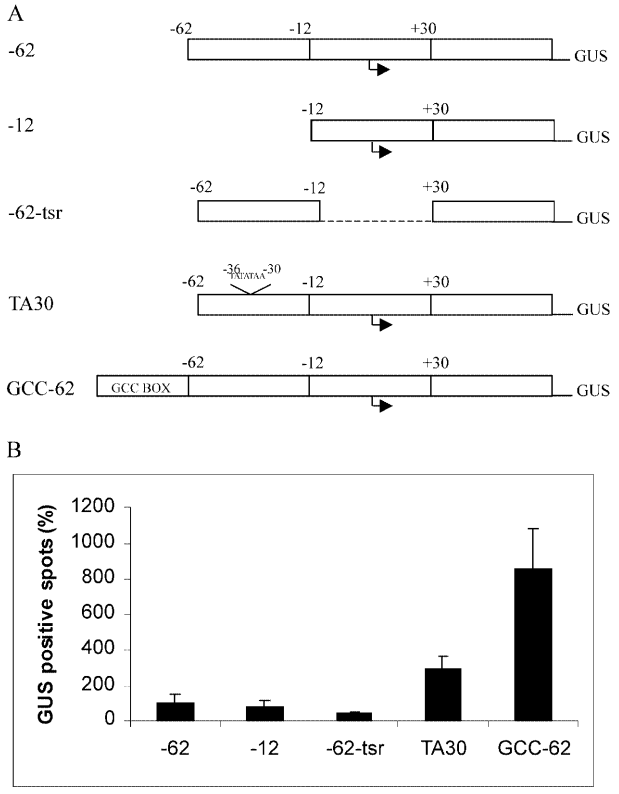

Fig. 2A, B Promoter activity of core promoter constructs with various deletions and mutations. **A** Schematic diagram of the chimeric constructs. The *numbers above the bars* indicate deletion end points relative to the transcription initiation site (+1) of the tCUP. The position of the transcription start site is indicated by an *arrow*. The *dotted line* indicates the sequence that was deleted. **B** The number of GUS-positive spots observed in the tobacco leaves was determined after particle gun delivery of the constructs shown in **A**. The number of spots is normalized to that observed with the –62-GUS construct. Each value represents the average of five independent experiments. The error bars indicate the standard error of the mean

–36 in the –62-GUS construct by a TATA box (TATATAA) increased the activity of the –62 core promoter by about three-fold. Addition of GCC-box sequences (Hart et al. 1993; Ohme-Takagi and Shinshi 1995) increased the activity of the –62 core promoter activity by about eight-fold. Similar results were also obtained with a transient expression assay in tomato protoplasts (data not shown).

The 135-bp fragment interacts with DNA-binding proteins

Electrophoretic mobility-shift assays were performed to investigate the possibility that nuclear proteins might interact with the tCUP promoter fragment extending from –197 to –62. The DNA fragment was ^{32}P end-labeled with the polynucleotide kinase and then incubated with nuclear extract prepared from tobacco leaves. As shown in Fig. 3, one major shifted band was observed in the lane containing nuclear extract compared with the control. In-

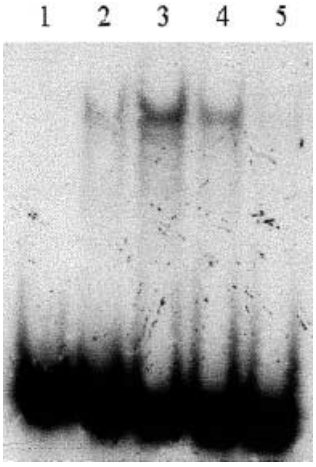

Fig. 3 Interaction of nuclear proteins from tobacco leaves with the 135-bp tCUP promoter fragment extending from –197 to –62. The 135-bp fragment was radiolabeled and 0.5 ng of the probe was incubated with nuclear proteins for 30 min at room temperature. The DNA-protein complexes were fractionated on a non-denaturing 5% polyacrylamide gel at 4°C for 2 h at 150 V. The gels was dried in a gel dryer and autoradiographed at –80°C. Lane 1, control reaction without nuclear extract; lanes 2 and 3, reactions containing 5 and 10 μg of nuclear extract, respectively; lanes 4 and 5, reactions with 10 μg of nuclear extract, plus s 10- and 100-fold excess of the unlabelled 135-bp fragment, respectively

clusion of a 10- or 100-fold excess of the unlabeled DNA fragment in the banding reaction significantly reduced formation of the shifted band, suggesting that the band is due to the binding of (a) sequence-specific protein(s), which is consistent with the functional identification of this region as an enhancer element.

Interaction with the –46 core promoter of CaMV 35S

To determine whether the enhancers within the –197 to –62 fragment could function with other core promoters, the fragment was also fused to the –46 core promoter of CaMV 35S (Fig. 4A). As shown in Fig. 4B, insertion of the fragment in both the forward and reverse orientation increased GUS activity by about 15-fold over the CaMV 35S core promoter activity in leaves of transgenic *Arabidopsis*. Insertion of two copies enhanced GUS activity by 40-fold. The data suggested that the –197 to –62 fragment could function as a transcriptional enhancer element and complement the 35S core promoter. In transgenic *Arabidopsis*, the average GUS activity for the –197-35S-GUS construct was about six times lower than that seen with the –197tCUP-GUS construct (data not shown). This can be explained by the fact that the tCUP promoter has a translation enhancer in the 5′ untranslated region (unpublished data).

Expression patterns in *Arabidopsis*

Histochemical staining for GUS activity was carried out to assess tCUP promoter activity in different organs. As

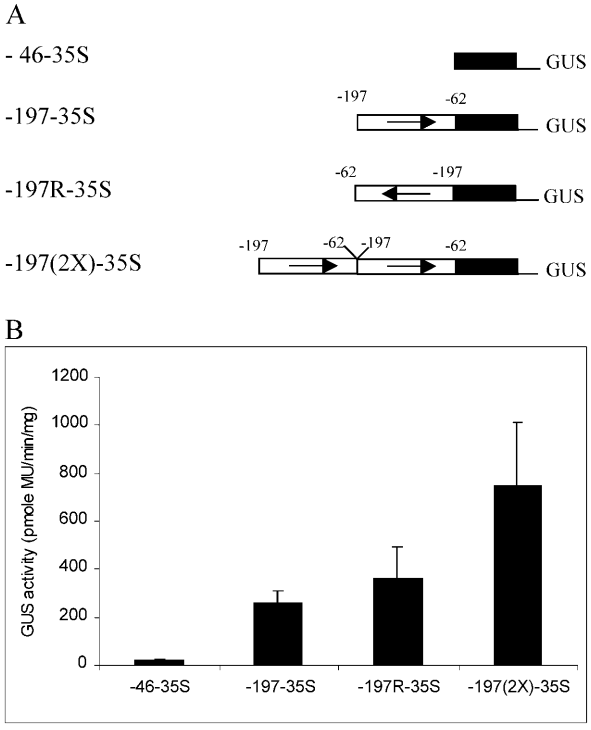

Fig. 4A, B Effect of the tCUP upstream region –197 to –62 on the activity of the –46 minimal CaMV 35S promoter. **A** Schematic diagram of the chimeric constructs. The *numbers above the bars* indicate deletion end points relative to the transcription initiation site. The *open boxes* represent the tCUP sequence and the *filled boxes* represent the CaMV 35S promoter sequence. **B** Average GUS specific activity (pmol MU/min/mg protein) in transgenic *Arabidopsis* plants containing the constructs shown in **A**. In all, 15–20 independent transgenic plants were analyzed for each construct. The error bars indicate the standard error of the mean

shown in Fig. 5, the GUS gene was expressed in leaves, stems, flowers and siliques of transgenic *Arabidopsis* plants containing *35S-GUS-NOS* (Fig. 5A) and *tCUP-GUS-NOS* (Fig. 5B). Both 35S and tCUP promoters exhibited constitutive expression when introduced into *Arabidopsis* plants. This data confirmed previous observations in tobacco (Foster et al. 1999).

To determine if the –197tCUP promoter fragment possessed all of the elements needed to drive constitutive expression in transgenic plants, histochemical analysis of GUS activity was performed in *Arabidopsis* plants transformed with the –197tCUP-GUS construct. As shown in the Fig. 5C, GUS activity was detected in leaves, stems and flowers of transgenic *Arabidopsis* plants containing the –197tCUP-GUS construct.

Transgenic *Arabidopsis* plants containing composite promoters in which the –197 to –62 fragment was fused to the –46 minimal promoter of CaMV 35S were also analyzed by histochemical GUS assay. The transgenic plant with the –46 minimal 35S promoter alone showed no GUS staining (Fig. 5D). Strong GUS staining was detected in the transgenic plants carrying constructs containing the –197 to –62 fragment fused to the –46 minimal 35S promoter in both forward and reverse orientation (Fig. 5E and F). GUS expression was detected in the leaves, stems, roots, flowers and siliques of transgenic *Arabidopsis* plants (Fig. 5G, H and I), suggesting that the sequence between –197 and –62 contained enhancer elements that are sufficient to drive constitutive expression.

Discussion

We have previously described the cloning of a cryptic promoter, tCUP, generated in a promoter trap experiment by T-DNA tagging of tobacco (Foster et al. 1999). Here we examined the components of the cryptic promoter and compared its activity and organization with that of the CaMV 35S promoter. The elements essential for constitutive expression of the tCUP promoter were located within the –197 to –62 fragment. Deletion of the 5′ sequences from –2054 to –394 relative to the transcription initiation site did not affect promoter activity significantly, suggesting that these sequences were not required for gene expression. Deletion of progressively larger segments of the tobacco tCUP promoter revealed enhancer elements in the –197 to –62 fragment. Duplication of the tCUP upstream region –197 to –62 increased promoter activity. It was previously reported that reiteration of the upstream region of CaMV 35S promoter can also enhance promoter activity (Fang et al. 1989; Comai et al. 1990). When the tCUP upstream region within fragment –197 to –62 was fused to the –46 minimal promoter of CaMV 35S, the basal level of expression increased by about 15-fold, and duplication of this fragment enhanced GUS activity by 40-fold. The –197 to –62 fragment enhanced the activity of the –46 minimal 35S promoter when inserted in both the forward and reverse orientation, indicating that this fragment can act as a transcriptional enhancer. Furthermore, the 135-bp fragment (from –197 to –62) of the tCUP promoter was shown to be sufficient to confer constitutive GUS expression on a –46 minimal CaMV 35S promoter in transgenic *Arabidopsis* plants. An electrophoretic mobility-shift assay showed that nuclear proteins prepared from tobacco leaves interact with the 135-bp fragment. We have delineated the sequence that is important for the binding of nuclear proteins, which will be published elsewhere.

Core promoter regions play a critical role in transcription by binding the preinitiation complex. Most eukaryotic gene promoters contain a TATA element, and mutations in this sequence result in severely reduced transcription efficiency in vivo and in vitro (Zhu et al. 1995; Yamaguchi et al. 1998). A second type of core promoter element, called an initiator (Inr), has also been found to anchor the preinitiation complex (Javahery et al. 1994; Schweizer and Mosinger 1994; Zhu et al. 1995; Smale 1997). In the absence of a TATA box, Inr can facilitate the initiation of transcription (Weis and Reinberg 1997). Constitutively active plant promoters that lack TATA boxes include the tobacco *eiF-4A*

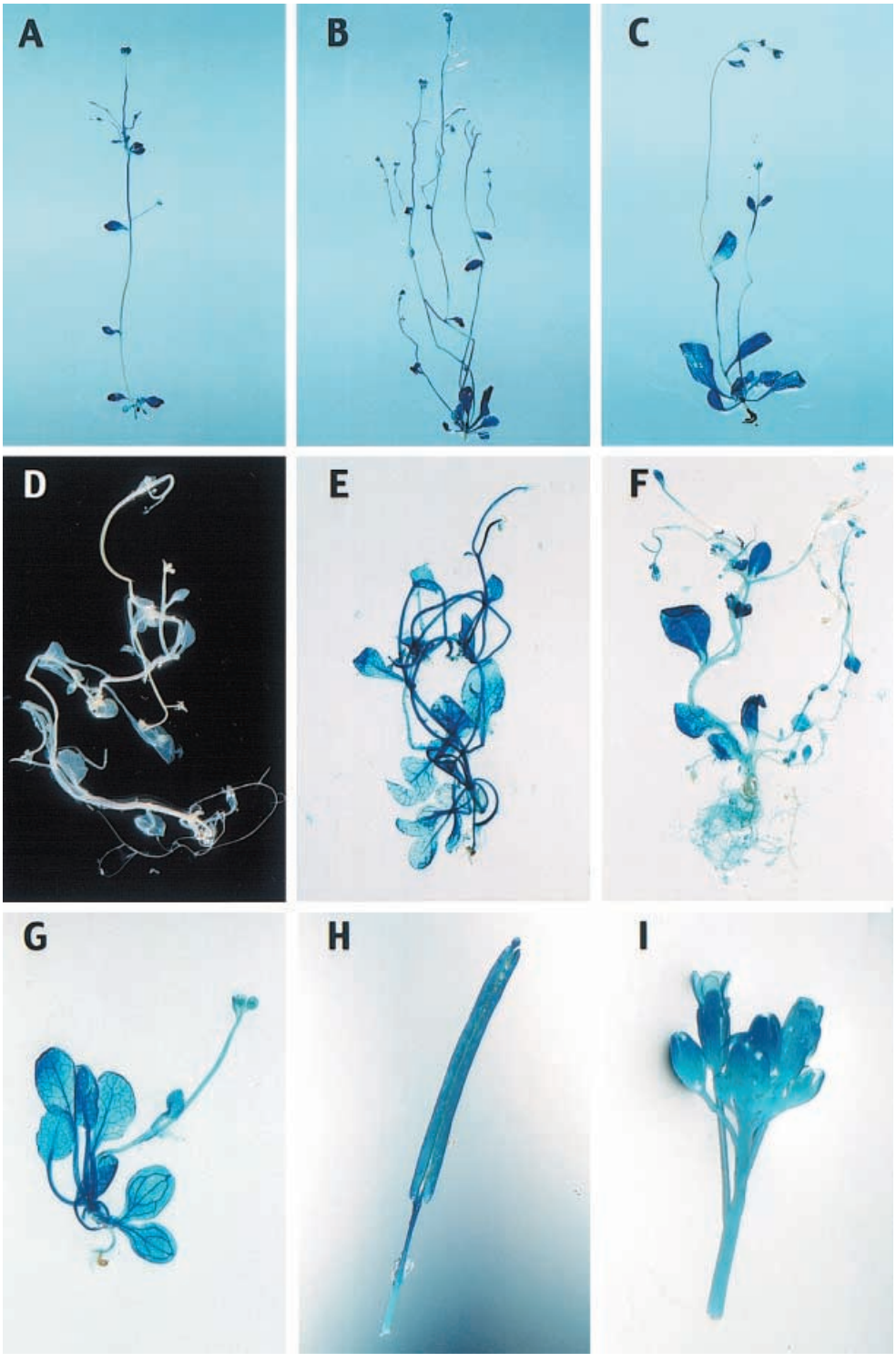

Fig. 5A–I Histochemical analysis of GUS expression in transgenic *Arabidopsis* plants containing the 35S-GUS construct (A), the tCUP-GUS construct (B), the –197-GUS construct (C), the –46 minimal 35S promoter and GUS fusion (–46–35S-GUS) (D), the –197 to –62 fragment fused with –46–35S-GUS (–197–35S-GUS) (E), the –197 to –62 fragment fused with –46–35S-GUS in reverse orientation (–197R-35S-GUS) (F), and in a seedling (G), siliques (H) and flowers (I) of transgenic plants bearing –197–35S-GUS

promoter (Mandel et al. 1995) and the maize *ZMDJ1* promoter (Baszczynski et al. 1997). The tCUP core promoter also lacks the TATA consensus sequence, but the sequence surrounding the transcription start site of tCUP has sequence similarity with the Inr element (Foster et al. 1999). Deletion of this sequence results in a significant drop in promoter activity, suggesting that the Inr element is functional in the tCUP core promoter and may play an important role in binding the preinitiation complex. However, we cannot exclude the possibility that a deletion between positions –12 and +30 is, in general, detrimental and not related to an Inr sequence. Replacement of the –30 to –36 sequence with a TATA consensus sequence (TATATAA) in the –62-GUS core

promoter construct increased the core promoter activity, indicating that the transcriptional activity of a TATA-less promoter could be substantially improved by introducing a TATA-box. Similar results have been observed in the study of mammalian TATA-less promoters (Weis and Beinberg 1997), revealing the similarity of the core elements between plants and animals.

The GCC-box enhancer element (Hart et al. 1993; Ohme-Takagi and Shinshi 1995) was investigated to determine if it could interact with the core promoter of tCUP to further increase the rate of transcription initiation. The GCC-box acts as the binding site for the ethylene-responsive element binding proteins (EREBP) and has been identified in the promoters of a number of pathogenesis-related genes (Zhou et al. 1997). It was shown that the addition of the GCC element to the minimal CaMV 35S promoter increased GUS expression (Ohme-Takagi and Shinshi 1995; Ishige et al. 1999). Our results indicate that GCC-box sequences can interact with the tCUP core promoter, and increased the promoter activity by about eight-fold. The data show that the promoter elements of plant genes and cryptic elements can be exchanged to create modular promoters.

Our previous studies suggested that cryptic gene regulatory elements might be abundant in the plant genome (Fobert et al. 1994; Foster et al. 1999). This was supported by the finding that the first two promoters identified by T-DNA promoter-trap experiments, pT218 and tCUP, were not associated with expressed genes (Fobert et al. 1994; Foster et al. 1999). Others have since isolated additional cryptic promoters from plants (Ökrésza et al. 1998; Plesch et al. 2000). The cryptic promoter T218 is expressed in a cell-specific manner (Fobert et al. 1994). It has a core promoter containing a TATA box and upstream activating sequences that regulate the spatial and developmental patterns of expression in seed coats. The constitutive cryptic promoter tCUP, however, lacks the TATA-box consensus sequence but possesses an Inr sequence and has enhancers that confer a constitutive pattern of gene expression. Our studies indicate that cryptic gene regulatory elements are as diverse and efficient as those associated with expressed genes and are organized in a similar fashion. Furthermore, we now demonstrate that enhancers and core promoter elements are interchangeable between cryptic promoters and promoters associated with expressed genes. This provides evidence that cryptic promoters function through the same basic regulatory mechanism. It therefore follows that they will modify the patterns of gene expression if rearrangements occur in the genome that bring them close to gene sequences. Further investigation of cryptic *cis*-elements and *trans*-acting factors will increase our knowledge of transcription regulation and its impact on evolution.

Acknowledgement We thank Dr. Thérèse Ouellet and Dr. Mark Jordon for critical reading of the manuscript. This research was funded in part by the Matching Investment Initiative Program at Agriculture and Agri-Food Canada. ECORC publication number 001574.

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