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A tobacco bZip transcription activator (TAF-1) binds to a G-box-like motif conserved in plant genes

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Tobacco nuclear extract contains a factor that binds specifically to the motif I sequence (5'-GTACGTGGCG-3') conserved among rice *rab* genes and cotton *lea* genes. We isolated from a tobacco cDNA expression library, a partial cDNA clone encoding a truncated derivative of a protein designated as TAF-1. The truncated TAF-1 (M_r = 26 000) contains an acidic region at its N-terminus and a bZip motif at its C-terminus. Using a panel of motif I mutants as probes, we showed that the truncated TAF-1 and the tobacco nuclear factor for motif I have similar, it not identical, binding specificities. In particular, both show high-affinity binding to the perfect palindrome 5'-GCCACGTGGC-3' which is also known as the G-box motif. TAF-1 mRNA is highly expressed in root, but the level is at least 10 times lower in stem and leaf. Consistent with this observation, we found that a motif I tetramer, when fused to the −90 derivative of the CaMV 35S promoter, is inactive in leaf of transgenic tobacco. The activity, however, can be elevated by transient expression of the truncated TAF-1. We conclude from these results that TAF-1 can bind to the G-box and related motifs and that it functions as a transcription activator.

Key words: G-box binding proteins/plant bZip proteins/plant histone promoters/rice *rab* promoters/*trans*-activators

Introduction

Recent investigations into plant genes have focused on sequence-specific DNA-binding proteins that may play a role in *trans* regulation. These DNA-binding proteins are usually localized in the nuclei, and their target DNA sequences and binding specificities can be characterized by gel mobility-shift assays and DNase footprinting techniques using either whole cell or nuclear extracts. Results from such *in vitro* experiments have led to the conclusion that 5' upstream regions of plant genes contain binding sites for multiple nuclear protein factors (cf. Allen *et al.*, 1989; Gilmartin *et al.*, 1990; Schindler and Cashmore, 1990). Moreover, in some cases, a single nuclear factor may interact with more than one promoter. For example, the tobacco nuclear factor, Activation Sequence Factor (ASF)-1, was first identified by its ability to bind to the TGACG motifs located in the −83 to −63 region of the cauliflower mosaic virus (CaMV) 35S promoter (Lam *et al.*, 1989). Further analyses, however, demonstrated that it also binds to a similar motif in the 5' regions of the wheat histone H3 gene (Katagiri *et al.*, 1989), nopaline synthase gene (Bouchez *et al.*, 1989; Katagiri *et al.*, 1989; Lam *et al.*, 1990), octopine synthase gene (Fromm *et al.*, 1989; Tokuhisa *et al.*, 1990), and the TR 1' and 2' promoters of octopine T-DNA (Bouchez *et al.*, 1989). These biochemical results are consistent with previous genetic data indicating that a regulatory gene may control the activity of several structural genes (cf. Coe and Neuffer, 1977).

Another nuclear factor, the G-box binding factor (GBF), also appears to bind to several classes of promoters. Giuliano *et al.* (1988) first reported that this factor binds to the G-box motif, 5'-TCTTACACGTGGCAYY-3' conserved in the upstream sequences of several dicotyledonous *rbcS* genes. A G-box-related motif, containing the core sequence CACGTG is also present in the 5' regions of two other classes of light-responsive genes: the *Arabidopsis cab* genes (Ha and An, 1988) and the chalcone synthase genes (*chs*) of *Petroselinum crispum* (Schulze-Lefert *et al.*, 1989a,b) and *Antirrhinum majus* (Staiger *et al.*, 1989). Staiger *et al.* (1989) have demonstrated that the G-box motif in *chs* is related to that of *Nicotiana tabacum rbcS* since the two sequences appear to compete for the same tobacco nuclear factor, CG-1. Their results suggest that CG-1 and GBF have similar binding specificities; however, it is not known whether the two protein factors are indeed identical or just related. It should be noted that the G-box or related motif is not exclusively associated with light-responsive genes because it is also found at −110 of the patatin (PI-II) promoter (Rosahl *et al.*, 1986) and at −200 of the *Arabidopsis* alcohol dehydrogenase (*Adh*) promoter (McKendree *et al.*, 1990; DeLisle and Ferl, 1990). These findings raise the possibility that GBF or CG-1 may simply be a ubiquitous factor capable of interacting with promoters of diverse regulatory properties.

Our laboratory is interested in the characterization of *trans*-acting factors that display sequence-specific binding to defined *cis* elements. We have previously described a rice nuclear factor that binds specifically to a 5'-GTACGTGGCG-3' sequence of the rice *rab16A* promoter (Mundy *et al.*, 1990). This sequence, designated as motif I, is conserved not only among all the four ABA-responsive *rab16* genes (A−D; Mundy and Chua, 1988; Yamaguchi-Shinozaki *et al.*, 1990), but also in cotton genes (*lea*) that are expressed during late embryogenesis (Baker *et al.*, 1988). Comparison of motif I and the G-box motif reveals extensive sequence homology. Here, we show that tobacco nuclear extract contains a factor which binds to the *rab16* motif I and that this binding is sensitive to competition by a G-box motif. We have isolated from a tobacco cDNA expression library, a partial cDNA clone encoding a truncated protein (TAF-1) with similar DNA-binding specificities to the nuclear motif I factor. Both the nuclear factor and the truncated TAF-1 show very high affinity binding to the G-box motif. TAF-1 contains an acidic domain at its N-terminus and at its C-terminus a basic domain

contiguous to a leucine repeat; moreover when expressed transiently in leaf cells, it can *trans*-activate a β-glucuronidase (GUS) reporter gene linked to a motif I tetramer.

Results

A tobacco factor binds to the conserved motif I, GTACGTGGCG

The upstream regions of rice *rab16* genes (*A, B, C* and *D*) contain a conserved sequence called motif I which serves as a binding site of rice nuclear proteins (Mundy *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1990). This motif is also found in the upstream regions of other ABA-responsive genes (see Skriver and Mundy, 1990 for a review). In the case of *rab16B*, motif I is located within a 70 bp region which spans from −275 to −206 (Figure 1A). When this 70 bp fragment was used as a probe in gel mobility-shift assays using nuclear extracts prepared from tobacco leaves, two complexes designated as I and II in the order of increasing mobility were seen (Figure 1B). To see whether one of the complexes resulted from specific interaction with motif I, we synthesized tetramers of motif I and its mutant derivative (Figure 1A) and used them as competitors in gel mobility-shift assays. Figure 1B shows that the slower-migrating complex I, was sensitive to competition by the wild type but not the mutant tetramer. These results indicated that nuclear extracts of tobacco leaves contain a factor which specifically binds to the conserved motif I of rice *rab16B*.

Isolation of a cDNA clone encoding a protein binding to motif I

We screened a tobacco leaf cDNA expression library using the 70 bp fragment of *rab16B* (Figure 1A) as a probe. One positive clone, 5a, was obtained from screening of 500 000 recombinant phages. The cDNA insert of 5a was subcloned into SK(−) plasmid and the recombinant plasmid p5a was used for further experiments.

The entire nucleotide sequence of the cDNA insert was determined by the dideoxy method (Figure 2A). The insert contains a partial cDNA of 1345 bp encoding an open reading frame of 265 amino acids, starting with an alanine which we have tentatively designated as the first amino acid residue. Analysis of the deduced amino acid sequence showed that the encoded protein contains at its C-terminus, six leucine residues (Nos 222, 229, 236, 243, 250 and 257) arranged as heptad repeats. In addition, a stretch of basic amino acids (residues 196 to 215) is located adjacent to the N-terminus of the leucine repeats. These two structural motifs, the basic domain and the leucine zipper, are characteristic features of a class of transcription factors referred to as the bZip proteins (Vinson *et al.*, 1989). It has been shown that for this group of proteins, the basic domain is involved in DNA binding (Talanian *et al.*, 1990), while the leucine repeats are involved in dimerization (O'Neil *et al.*, 1990). Another distinctive feature of the encoded protein is that its N-terminal region, from Ala1 to Pro107, has a net negative charge of 7. This region also contains a high proportion of serine and threonine. Domains of *trans* factors enriched in acidic residues and or hydroxy amino acids have been implicated in transcription activation. For convenience, we have designated the protein encoded by this partial cDNA as truncated TAF-1.

To determine how many genes in the tobacco genome are

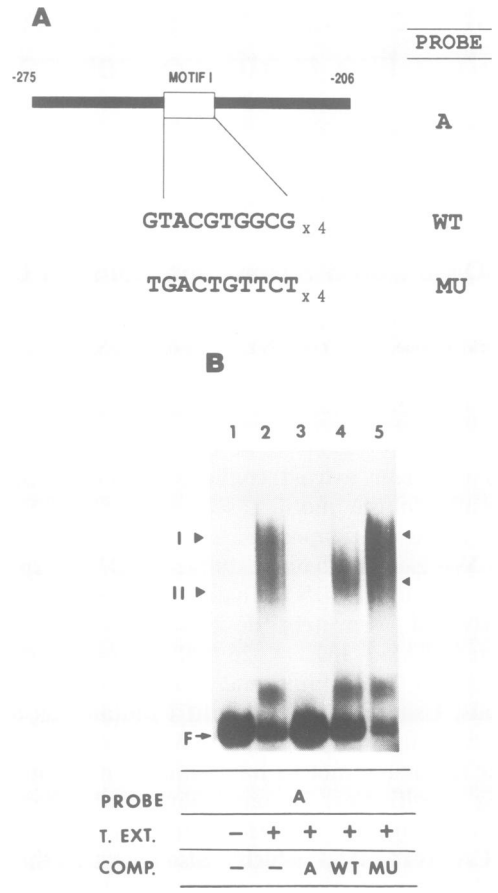

Fig. 1. Tobacco nuclear extracts contain a binding activity specific for motif I of rice *rab16* genes. **(A)** DNA probes used for gel mobility-shift assays. Probe A which is 70 bp long contains the sequences between −275 and −206 of the rice *rab16B* gene (Yamaguchi-Shinozaki *et al.*, 1990). The wild type probe contains four tandem copies of motif I, CTACGTGGCG and the mutant (MU) probe contains four tandem copies of a mutant sequence of motif I in which the Gs were changed to Ts and the Ts were changed to Gs. **(B)** Gel mobility-shift assays of tobacco nuclear extract using probe A. Experiments were performed as described in Materials and methods. Competitors were added to 300-fold molar excess. F, free probe; I and II, complexes I and II, respectively; T. Ext., tobacco nuclear extract; comp., competitors. F, free probe. Arrowheads indicate positions of specific DNA−protein complexes.

related to the TAF-1 gene, we carried out Southern blot hybridizations using the 1.2 kb *Eco*RI fragment of the TAF-1 partial cDNA as a probe. Two hybridizing bands were obtained with genomic DNA digested with either *Hin*dIII or *Eco*RI (data not shown). These results suggest that TAF-1 is likely to be encoded by one or two genes.

DNA-binding specificity of truncated TAF-1

To see whether the protein product encoded by p5a could indeed bind DNA, we prepared extracts from *Escherichia coli* carrying the expression vector pSK(−) before and after IPTG induction. In this vector, the Met22 of the partial TAF-1 coding sequence was presumably used as the initiator methionine to produce an N-terminal truncated TAF-1 of M_r ~26 000. The extracts were tested with motif I wild type and mutant tetramers in gel mobility-shift assays. Figure 2B shows that the wild type tetramer formed specific complexes when incubated with extracts from IPTG-induced cells (lane 2), but not with extracts from uninduced cells

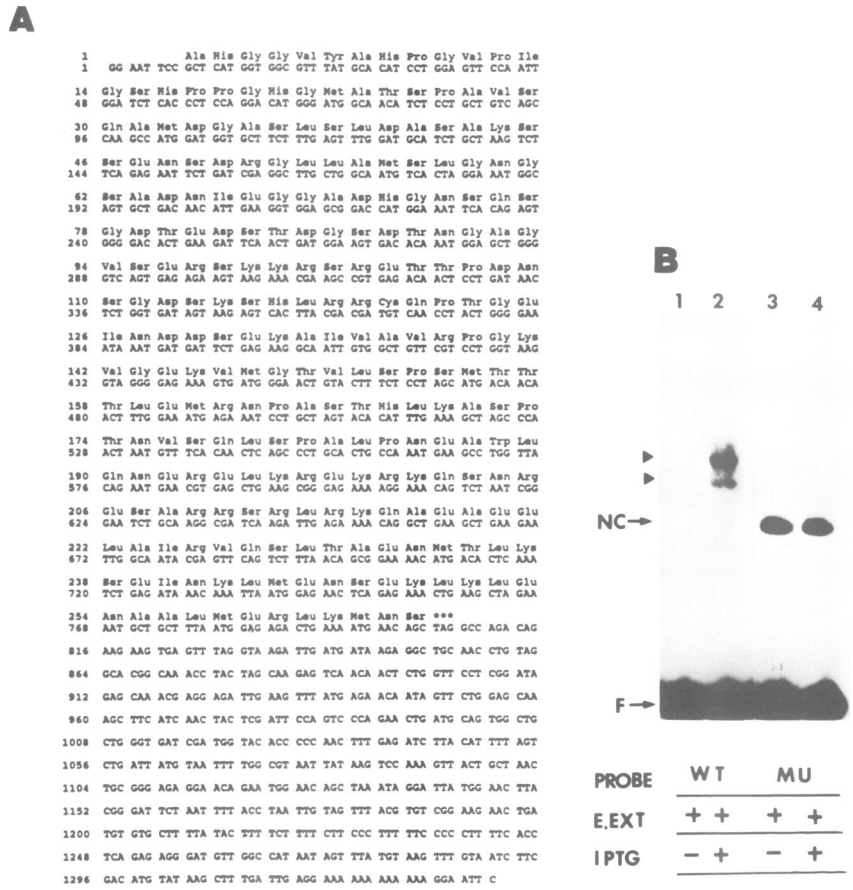

Fig. 2. Characterization of the cDNA clone 5a and its encoded product (TAF-1). (**A**) Nucleotide sequence of clone 5a. The partial cDNA is comprised of 1345 bp and contains an open reading frame of 264 amino acids. The first methionine is located at position 22. (**B**) DNA-binding specificity of the protein product encoded by clone 5a. The partial cDNA clone was placed downstream of the *lacZ* promoter in the vector pSK(−) and the recombinant plasmid was transformed into *E.coli*. In this expression vector, the Met22 was presumably used as the initiator methionine to produce a truncated TAF-1 of M_r ~26 000. Exponential phase cultures were induced with 2 mM IPTG (+); uninduced cultures were used as controls (−). Extracts (E. ext) were prepared from induced (+) and uninduced (−) cultures and fractionated with ammonium sulfate as described in Materials and methods. SDS−PAGE analysis showed the presence of a 26 000 kd polypeptide in extracts from the induced, but not the uninduced cultures (data not shown). Gel mobility-shift assays were performed using the WT or the mutant motif I tetramer as a probe (Figure 1A). F, free probe; NC, non-specific complex; arrowheads indicate specific complexes.

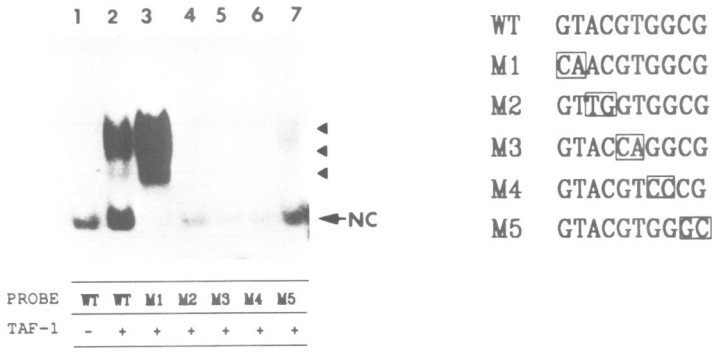

Fig. 3. Mutational analysis of motif I. Tetramers of WT motif I and of various mutants (M1−M5) were assayed for their ability to interact with the recombinant TAF-1 produced in *E.coli* by gel mobility-shift. The mutants contained successive 2 bp alterations as indicated in the figure. NC, non-specific complex; arrowheads indicate specific complexes.

(lane 1). Neither extracts gave any specific complexes with the mutant tetramer (Figure 2B, lanes 3 and 4). These results indicate that the recombinant protein encoded by the p5a partial cDNA insert binds specifically to motif I and therefore, its full-length product, TAF-1, is a good candidate for the motif I factor. Because the wild type tetramer contains four copies of motif I, it was therefore not surprising that multiple complexes were obtained with this probe (Figure 2B, lane 2).

The band labeled NC (Figure 2B, lanes 3 and 4) is a non-specific complex between an unknown bacterial protein and the mutant tetramer probe. Because the formation of NC was not dependent on IPTG, this complex was not due to and did not require the recombinant TAF-1. The NC band can

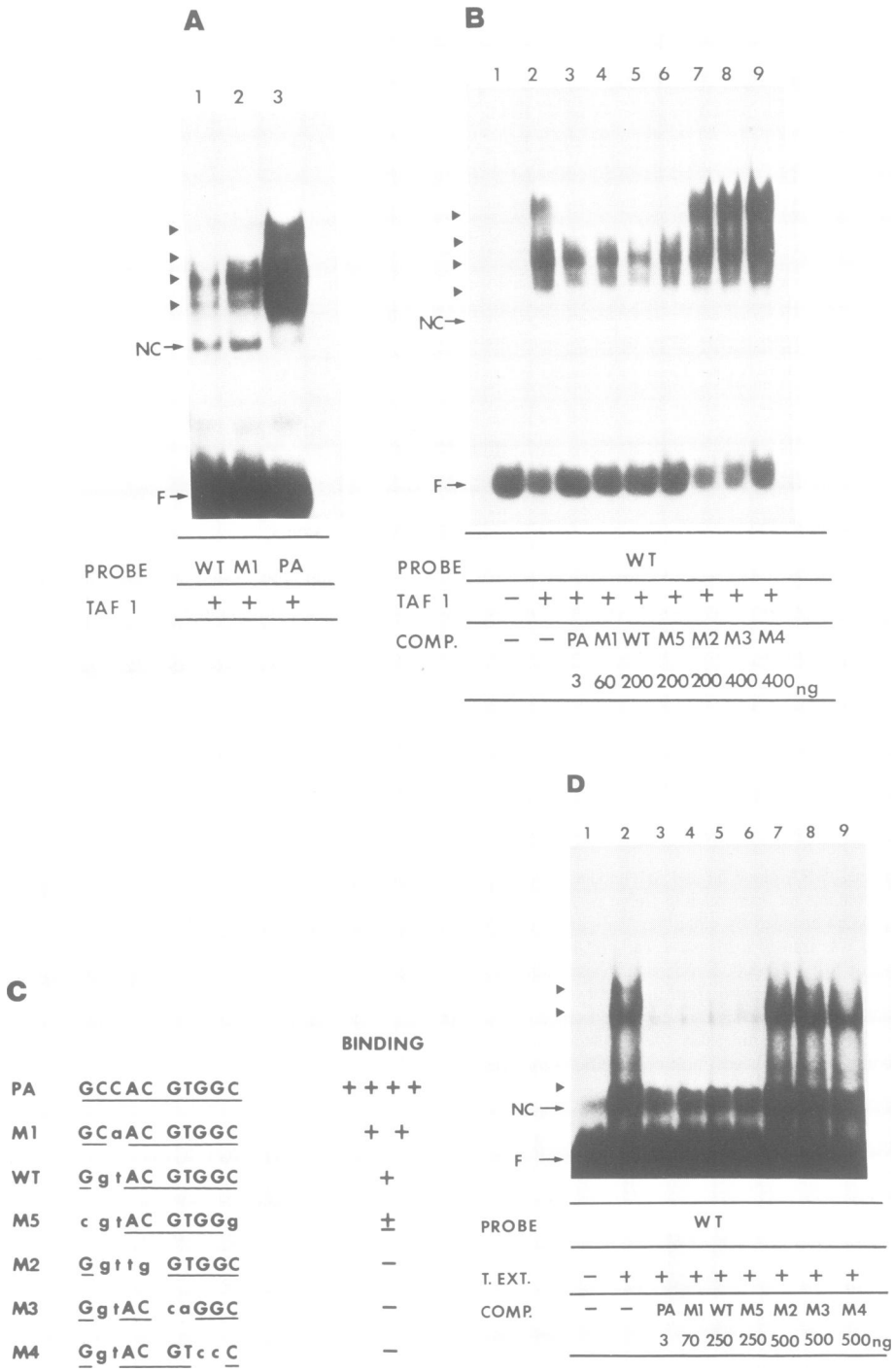

Fig. 4. DNA-binding site sequence specificities of the truncated TAF-1 and the nuclear motif I factor. (**A**) TAF-1 preferentially binds to a perfect palindromic sequence. Tetramers of WT motif I, the mutant M1 and the perfect palindrome (PA), GCCACGTGGC, were used as probes in gel mobility-shift assays with *E.coli* extracts containing the truncated TAF-1. F, free probe; NC, non-specific complex. The specific complexes, indicated with arrowheads, have a slower mobility than the non-specific complex. (**B**) Relative binding affinities of TAF-1 to motif I (WT) and related sequences. WT motif I tetramer was used as a probe in gel mobility shift assays with *E.coli* extracts containing the truncated TAF-1. Different concentrations of tetramers of mutants M1−M5, as well as the tetramer of the perfect palindromic sequence (PA) were tested for their ability to compete with the WT sequence for TAF-1. For WT, PA, M1 and M5, the concentrations that gave ~50% competition were used in the experiments shown here. Higher concentrations were used for mutants M2, M3 and M4. Comp., Competitor. (**C**) Nucleotide sequences of WT, mutants M1−M5, and the perfect palindrome, PA. In this figure, the WT motif I of the tetramer is shown with the TACGTG hexanucleotide as the core sequence. This 5′ nucleotide G of the motif is derived from the 3′ nucleotide of the preceding motif in the tetramer. Other sequences were represented accordingly. Nucleotide differences with PA are shown in lower case. Relative binding affinities to TAF-1 are indicated on the right. (**D**) Relative binding affinities of the nuclear motif I factor to motif I (WT) and related sequences. Experiments were carried out as in (B) except that tobacco nuclear extracts (T. Ext.) were used. Note the slight differences in the concentrations of competitors between (B) and (D). F, free probe; NC, non-specific complex; arrowheads indicate specific complexes.

(A)

TAF-1	(196-215)	K R E K R K Q S N R E S A R R S R L R K
EmBP-1	(105-125)	K R E R R K Q S N R E S A R R S R L R K
HBP-1	(254-274)	K K Q K R K L S N R E S A R R S R L R K
OCSBF-1	(26-46)	R R E K R R L S N R E S A R R S R L R K
O2	(228-248)	R V R K R K E S N R E S A R R S R Y R K
TGA-1a	(87-107)	K V L R R L A Q N R E A A R K S R L R K
TGA-1b	(185-205)	K K R A R L V R N R E S A Q L S R Q R K

(B)

		*	*	*	*	*	*
TAF-1	(216-258)	QAEAEEL	AIQVQSL	TAENNTL	KSEINKL	MENSEKL	KLENAAL
EmBP-1	(126-168)	QQECEEL	AQKVSEL	TAANGTL	RSELDQL	KKDCKTM	ETENKQL
HBP-1	(275-317)	QAECEEL	GQRAEAL	KSENSSL	RIELDRI	KKEYEEL	LSKNTSL
OCSBF-1	(47-68)	QQHLDEL	VQEVARL	QADNARV			

Fig. 5. Comparison of amino acid sequences of the basic domains and leucine repeats of plant bZip proteins. (**A**) Basic domains. TAF-1 (this paper); EmBP-1, Guiltinan *et al.*, 1990; HBP-1, Tabata *et al.*, 1989; OCSBF-1, Singh *et al.*, 1990; O2, Hastings *et al.*, 1989; Schmidt *et al.*, 1990; TGA1A and TGA1B, Katagiri *et al.*, 1989. Conserved amino acid residues are boxed. (**B**) Leucine repeats. For references, see (A). Repeated leucine residues are marked with asterisks.

also be seen with the WT tetramer probe (cf. Figures 3 and 4A), but its intensity varied between experiments and was very faint in the experiment presented in lanes 1 and 2 of Figure 2B.

Binding site sequence specificity of TAF-1

To define nucleotides in motif I critical for interaction with TAF-1 we synthesized a set of motif I mutant tetramers (M1−M5) which contain successive 2 bp substitution mutations. The ability of these mutants to bind to truncated TAF-1 was assessed by gel mobility-shift assays. Because the truncated TAF-1 contains the entire bZip domain, in these experiments we assumed that its DNA-binding specificity is indistinguishable from that of the full-length product. We found that mutations in the central six nucleotides of motif I (M2, M3 and M4) virtually abolished binding and mutations in the last two nucleotides (M5) severely reduced TAF-1 binding (Figure 3). By contrast, mutant M1 which contains mutations in the first two nucleotides, showed an increased affinity for the same factor.

Detailed analysis of the M1 sequence revealed that it shares nine nucleotides with the 10-nucleotide palindromic sequence (PA) GCCACGTGGC (Figure 4C), which is identical to the G-box motif found at −190 of the petunia *rbcS-611* gene (Tumer *et al.*, 1986). Because bZip proteins bind to their target DNA sites as dimers, it is reasonable to assume that TAF-1 might show a preference for palindromic sequences. To examine this possibility, we investigated the interaction of PA and the truncated TAF-1 by gel mobility-shift assay. We found that the PA tetramer indeed showed a higher affinity for TAF-1 than the wild type tetramer (Figure 4A).

To assess the relative affinities of wild type (WT), PA and M1−M5, for TAF-1 we used the wild type tetramer as a probe and increasing amounts of unlabeled PA or mutant tetramers as competitors (data not shown). Figure 4B shows that ~50% competition was obtained with 200 ng of wild type while only 60 ng of M1 and 3 ng of PA were sufficient to give the same degree of competition. These results suggest that the binding affinity of TAF-1 for PA and M1 is ~66 and 3.3 times higher, respectively, than for WT. Mutant M5 was less effective in this competition assay than WT, in agreement with the results obtained by direct binding to the probe (Figure 3). The remaining mutants M2, M3 and M4

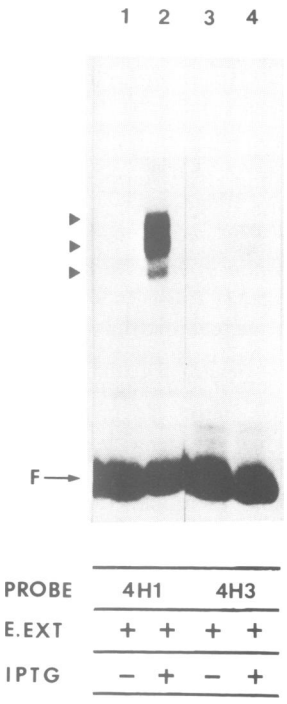

Fig. 6. TAF-1 binds to the *hex* motif of the wheat histone H3 promoter. *E.coli* extracts (E. ext) were prepared from IPTG-induced (+) and uninduced (−) cultures and fractionated with ammonium sulfate as detailed in Materials and methods. Gel mobility-shift assays were performed using tetramers of the WT (4H1) or the mutant (4H3) *hex* sequence (Katagiri *et al.*, 1989). WT, −180 TTCGGCCACGTC-ACCAATCCG −160; mutant, −180 TTCGGCCACGCGTCCA-ATCCG −160. Note that the three nucleotides at positions −168 to −170 have been changed to CGT from TCA. Specific complexes are indicated by arrows. F, free probe.

were ineffective as competitors at concentrations of 200 ng or higher.

The results obtained from the competition experiments (Figure 4B) are consistent with those obtained from direct binding (Figures 3 and 4A), and they are summarized in Figure 4C. Comparison of the relative binding affinities with the nucleotide sequences of PA, WT and the various mutants shows that the PA has the highest affinity for TAF-1.

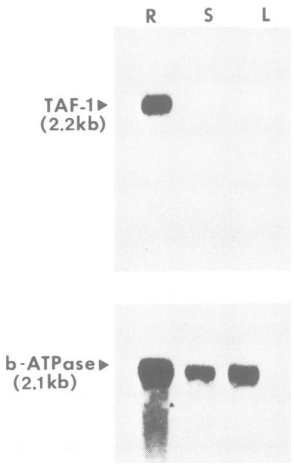

Fig. 7. Northern blot analysis of TAF-1 mRNA in different organs of the tobacco plant. poly(A) RNA (1 μg) from root (R), stem (S) and leaf (L) of tobacco plants were used. The hybridization probes were TAF-1 cDNA (**upper panel**) and β-ATPase cDNA (**lower panel**). For other details, see Materials and methods.

Moreover, the binding affinity appears to decrease with an increasing degree of nucleotide mismatch.

Binding site sequence specificity of the nuclear motif I factor

Although both the nuclear motif I factor (Figure 1B) and the recombinant TAF-1 (Figure 2B) showed sequence-specific binding to motif I, it was not known whether they are indeed the same factor. To investigate this point, we determined the binding site sequence requirement of nuclear motif I factor by gel mobility-shift assays using motif I as a probe and the panel of mutant tetramers (Figure 4C) as competitors. Within the limit of sensitivity of this technique the results obtained with the nuclear factor (Figure 4D) were about the same as those with the truncated TAF-1 (Figure 4B). We conclude from these results that the full-length TAF-1 is a good candidate for nuclear motif I factor or is a major component of it.

Truncated TAF-1 also binds to the hex motif

The basic region of TAF-1 is strikingly homologous to the corresponding region of HBP-1 (Figure 5A), a wheat DNA-binding protein which interacts with the conserved hexamer (*hex*) sequence located at −171 to the wheat histone H3 promoter (Tabata *et al.*, 1989). This observation prompted us to examine whether the recombinant TAF-1 would also bind to this sequence. Figure 6 shows that the truncated form of TAF-1 produced in *E.coli* could indeed bind to the −180 to −160 region of the wheat histone H3 promoter (lanes 1 and 2). The binding was dependent on the intact *hex* sequence since a 3 bp mutation in positions −168 to −170 greatly diminished the binding (lanes 3 and 4).

Expression pattern of TAF-1 mRNA

Figure 7, top panel, shows that the TAF-1 mRNA is expressed in roots but is undetectable in stems and leaves. A longer exposure of the same autoradiogram, however, revealed a faint band of the same size in these two organ samples (data not shown). We estimated that there is about 10−20 times more TAF-1 mRNA in roots than in stems and leaves. As a control, the mRNA for the constitutively expressed β-ATPase gene (Boutry and Chua, 1985) is present at ~2-fold higher levels in roots than in the other two organs (Figure 7, bottom panel).

Because the size of the TAF-1 mRNA is 2.2 kb, we estimated that ~0.8 kb of TAF-1 sequences are missing from our partial cDNA clone which encodes the 3′ portion of the gene.

Table I. A truncated TAF-1 can *trans*-activate the expression of GUS reporter gene linked to a motif I tetramer

	(A) WT × 4		(B) MU × 4	
	−ABA	+ABA	−ABA	+ABA
No bombardment	250	383	237	230
Vector control	500	500	550	600
35S/partial TAF-1 cDNA	4300	4150	515	633

Adult leaves (~8 cm × 4 cm) from transgenic plants containing either the motif I tetramer fused to X-GUS90 (A) or the mutant tetramer fused to X-GUS90 (B) were cut into two sections. One section was bombarded with tungsten particles coated with pMON505 (Benfey *et al.*, 1989) while the other section with a pMON505 derivative containing a 35S cDNA 5a chimeric gene. The latter was comprised of the CaMV 35S promoter (−343 to +8), the partial TAF-1 cDNA and the *rbcS-E9* 3′ polyadenylation signal. After that the leaf sections were incubated in the dark in water or 10^{-4} M ABA for 24 h at room temperature. Non-bombarded leaf sections were used as controls. GUS activities were measured according to Jefferson *et al.* (1987) and expressed as pmol 4-methyl umbelliferone/mg protein/min. Results shown are representative of four independent experiments for (A) and three independent experiments for (B).

TAF-1 is a trans-activator

To see whether the recombinant TAF would function as a transcription activator *in vivo*, we synthesized double-stranded oligonucleotides containing either four copies of WT motif I sequence or four copies of a motif I mutant sequence. These tetramers were separately placed upstream of the −90 CaMV 35S promoter (Benfey *et al.*, 1989). In both cases, the bacterial β-glucuronidase (GUS) coding sequence was used as the reporter gene. These chimeric genes were transferred into tobacco and several independent transgenic plants for each construct were analyzed for GUS activity.

We found that the WT motif I tetramer conferred little or no activity in leaves of transgenic plants, while the mutant tetramer was inactive (Table I). Addition of ABA had no noticeable effect on either the GUS activity (Table I) or mRNA levels in leaves of transgenic plants harboring either construct (data not shown). Detailed expression pattern conferred by the WT motif I tetramer in different tissues and at different stages of development will be reported elsewhere (Salinas,J., Oeda,K. and Chua,N.-H., manuscript in preparation).

The low expression level in leaves conferred by motif I could be due to a reduced concentration of its cognate transcription activator in cells of this organ. This is also consistent with the low TAF-1 mRNA in leaf (Figure 7). If the truncated TAF-1 binds to motif I *in vivo* and functions as a *trans*-activator, it should be possible to elevate GUS expression in leaves by overexpression of TAF-1. To test this notion, we constructed a chimeric gene comprised of the CaMV 35S promoter (−343 to +8) and the partial

Table II. Recognition sequences of several plant DNA-binding proteins

DNA-binding protein	Recognition sequence	Position	Gene	Reference
GBF	G C C A C G T G T C	−253	Tomato *rbcS-3A*	Giuliano *et al.* (1988)
	T C C A C G T G G C	−236	*A.thaliana rbcS-1A*	
	T A C A C G T G G C	−228	Pea *rbcS-3,6*	
	C A G A C G T G G C	−240	*N.plumbaginifolia Cab-E*	Schindler and Cashmore (1990)
	G C C A C G T G G A	−213	*A.thaliana Adh*	McKendree *et al.* (1990)
CG-1	G T C A C G T G C C	−122	*A.majus chs*	Staiger *et al.* (1989)
	T C C A C G T G G C	−155	*P.crispum chs*	
	T A C A C G T G G C	−277	*N.tabacum rbcS*	
	G C C A C G T G A C	−57	Adenovirus major late promoter	
HBP-1	G T G A C G T G G C	−171	Wheat histone H3[a] gene	Tabata *et al.* (1989)
EmBP-1	G A C A C G T G G C	−147	Wheat Em	Guiltinan *et al.* (1990)
	G T G A C G T G G C	−171	Wheat histone H3[a] gene	
OCSBF-1	− T G A C G T A A −		OCS element concensus	Singh *et al.* (1990)
TAF-1	G G T A C G T G G C		Rice *rabA-D*	This paper
	G C C A C G T G G C	−190	Petunia *rbcS-611*	
	G T G A C G T G G C	−171	Wheat histone H3[a]gene	

[a]Sequence of the bottom strand DNA was shown.

TAF-1 cDNA coding sequence. Plasmid DNA containing this chimeric gene was introduced by high velocity bombardment into cells of transgenic leaves carrying the motif I tetramer−GUS transgene. Table I shows that bombardment of the 35S/TAF-1 effector plasmid indeed increased GUS expression in the leaves by ~10- to 15-fold while the vector DNA alone gave <2-fold stimulation. Because only a fraction of the leaf cells received the effector plasmid, it is likely that the actual amount of activation with the 35S/TAF-1 construct was higher. The GUS expression was dependent on the ability of TAF-1 to bind to motif I, since leaves of transgenic plants carrying the motif I tetramer failed to respond to the same effector plasmid. We also tested whether the GUS expression in leaves conferred by motif I and TAF-1 was influenced in any way by ABA treatment. Table I shows that the GUS activity was not significantly different between the ABA-treated and the control samples.

Discussion

TAF-1 is likely to be the tobacco nuclear motif I factor

In this paper we have shown that tobacco nuclear extract contains a factor which interacts specifically with the motif I sequence conserved in *rab* (Mundy and Chua, 1988; Yamaguchi-Shinozaki *et al.*, 1990) and *lea* (Baker *et al.*, 1988) genes. From a tobacco cDNA expression library we have isolated a partial cDNA clone p5a encoding a C-terminal portion of a protein designated as TAF-1. The truncated TAF-1 protein produced in *E.coli* has binding specificities very similar, if not identical, to the nuclear motif I factor when tested with a panel of discriminating probes (cf. Figure 4B and D). This result provides strong evidence that the full-length TAF-1 is the nuclear motif I factor or accounts for a part of its activity.

Structure of TAF-1

Nucleotide sequence analysis of the partial cDNA clone p5a shows that the truncated TAF-1 (M_r ~26 000) contains at its carboxy terminus a basic domain abutting a leucine repeat (Figure 2A). This bipartite structure is characteristic of the bZip class of DNA-binding proteins (Vinson *et al.*, 1989). So far, cDNA clones encoding five other plant bZip proteins have been isolated and characterized (Hastings *et al.*, 1989; Katagiri *et al.*, 1989; Tabata *et al.*, 1989; Schmidt *et al.*, 1990; Singh *et al.*, 1990). During the preparation of this manuscript, Guiltinan *et al.* (1990) described a partial cDNA clone encoding a wheat bZip protein which binds to a conserved sequence within a 75 bp ABA-responsive element. Amino acid sequence comparison between TAF-1 and these six other bZip proteins shows a high degree of homology only in the basic domain (Figure 5A). The most striking conservation is found among TAF-1, HBP-1 (Tabata *et al.*, 1989), EmBP-1 (Guiltinan *et al.*, 1990) and OCSBF-1 (Singh *et al.*, 1990). The basic domains of TAF-1 and EmBP-1 are virtually identical, with a single substitution of Lys for Arg at position 4. Since the basic domain of bZip proteins is involved in DNA recognition, this sequence conservation implies that the target DNA sequences of these DNA-binding proteins are likely to be similar. Table II shows that this is indeed the case for TAF-1, HBP-1 and EmBP-1.

The sequence homology between TAF-1 and EmBP-1 also extends in part, to the leucine zipper region (Figure 5B), raising the possibility that these two proteins may interact to form heterodimers.

TAF-1 can function as a trans-activator

Of the six plant bZip proteins described thus far, only two have been implicated in regulating transcription. There are genetic data demonstrating a requirement of the O2 gene product for the transcription of the 22 kd zein gene (Jones

et al., 1977). Based on this genetic evidence, O2 is likely to be a positive regulator. We have recently provided direct biochemical evidence that the tobacco bZip protein, TGA1a functions as an activator in a plant (Yamazaki *et al.*, 1990) as well as a HeLa *in vitro* transcription system (Katagiri *et al.*, 1990). Furthermore, purified TGA1a protein can activate transcription of the −90 CaMV 35S/GUS chimeric gene (Benfey *et al.*, 1989) when microinjected into leaf cells of transgenic tobacco harboring this reporter construct (Neuhaus,G., Neuhaus-Url,G., Katagiri,F., Seipel,K. and Chua,N.-H., unpublished data). This result indicates that TGA1a can also function as a transcription activator *in vivo*. A major point of our work here is the demonstration that TAF-1 is not only a DNA-binding protein, but also a transcriptional activator. We showed that a 35S truncated TAF-1 chimeric gene, when expressed transiently in tobacco leaf cells, can increase the expression of a GUS reporter gene linked to the WT motif I tetramer, but not the mutant motif I tetramer (Table I). Since TAF-1 binds to the WT but not to the mutant tetramer, our results demonstrate that TAF-1 can function as a *trans*-activator *in vivo*. In this connection, we note that the amino-terminal region (amino acids 1−86) of the truncated TAF-1 is acidic and therefore, may serve as a transcription activation domain (Ptashne, 1988).

Relationship of TAF-1 to GBF and CG-1

Although TAF-1 was originally isolated as a DNA-binding protein specific for motif I of *rab* genes, it binds with higher affinity to the perfect palindromic sequence GCCACGTGGC (Figure 4) which contains the hexanucleotide core CACGTG found in G-box and related motifs of several plant promoters (Table II). Because the palindromic sequence is identical to the G-box sequence of petunia *rbcS-611* gene (Tumer *et al.*, 1986) and differs from the *Arabidopsis rbcS-1A* G-box by only 1 bp (Table II) this result suggests that TAF-1 can also recognize other G-box sequences and related motifs.

Plant nuclear factors that recognize G-box and related motifs *in vitro* have been reported by several laboratories (Table II). The GBF of tomato and *Arabidopsis* binds to the G-box of tomato *rbcS-3A*, *Arabidopsis rbcS-1A*, pea *rbcS-3.6* (Giuliano *et al.*, 1988), and a similar motif, CAGACGTGGC, located at −240 of the *Nicotiana plumbaginifolia Cab-E* promoter (Schindler and Cashmore, 1990; Table II). Whole cell extracts of *Arabidopsis* cell cultures also contain GBF activity McKendree *et al.*, 1990), which binds to the G-box elements of *rbcS-1A* and *Adh*, but not to the related motif, GCCACGTGAC, found in the upstream activating element (UAE) of the adenovirus major late promoter (Table II). In tobacco and other higher plants, Staiger *et al.* (1989) have described a nuclear factor CG-1 which interacts with the G-box-like motif located in the upstream region of *A.majus chs* gene. This factor also binds to the *P.crispum chs* G-box, although with a lower affinity. CG-1 may be distinct from GBF because it binds to the UAE of the adenovirus major late promoter but not the *N.plumbaginifolia Cab-E* promoter (Table II). Taken together, the *in vitro* binding experiments using plant nuclear extracts are consistent with multiple G-box-binding factors that possess overlapping specificities.

The notion of a family of G-box-binding proteins in plants is supported by three additional lines of evidence. First, the GBF in *Arabidopsis* leaf nuclear extracts forms at least two complexes with G-box sequences (Giuliano *et al.*, 1988) which differ in mobility from that formed by the GBF of *Arabidopsis* cell culture (McKendree *et al.*, 1990). This result indicates that *Arabidopsis* may contain more than one GBF. Indeed, UV cross-linking experiments have shown that the *Arabidopsis* GBF activity can be attributed to at least two proteins of M_r 18 000 and 31 000 (DeLisle and Ferl, 1990). Second, the results obtained with recombinant DNA-binding proteins so far demonstrate that at least two different factors, TAF-1 and the recently described EmBP-1 (Guiltinan *et al.*, 1990), can bind to G-box-related sequences (Table II). In view of the conserved amino acid sequences in their basic domains (Figure 5A), we predict that HBP-1 and OCSBF-1 would also bind to the G-box and related motifs as well. This possibility should be tested by future experiments. Third, nuclear extracts of cauliflower contain at least three factors that recognize the CACGTG core motif but differ in their preferences for flanking sequences (M.Williams and N.-H.Chua, in preparation).

It is particularly important to point out that both TAF-1 and EmBP-1 bind to the *hex* motif, GTGACGTGGC, of the wheat histone H3 gene, which deviates from the perfect palindromic sequence by only 2 bp (Table II). At least three other proteins, the tobacco TGA1a and TGA1b (Katagiri *et al.*, 1989) and the wheat HBP-1 (Tabata *et al.*, 1989) can recognize the same *hex* sequence. Whether these three proteins would also interact with the G-box remains to be established.

The perfect palindromic sequence (PA), GCCACGTGGC, differs by only 1 bp from the sequence of the upstream activating element (UAE), GCCACGTGAC, located at −62 to −53 of the adenovirus major late promoter. The UAE can interact with two human transcription factors, USF and TFE-3, and full-length cDNA clones encoding these factors have been reported recently (Gregor *et al.*, 1990; Beckmann *et al.*, 1990). In contrast to TAF-1 which is a bZip protein, both USF and TFE-3 are c-myc-related proteins containing a helix-loop-helix (HLH) motif preceded by a basic domain which is presumably involved in DNA recognition. Notwithstanding the striking similarity in the nucleotide sequences of their recognition sites, there is no obvious homology in the amino acid sequences of the basic domains between TAF-1 and these two human transcription factors. Future experiments would determine whether there are any helix-loop-helix proteins in plants with specificities for G-box and related motifs and, conversely, whether there are any mammalian bZip proteins that would interact with UAE and related sequences.

Function of TAF-1 in vivo

Although we have shown here that the truncated TAF-1 can function as a transcriptional activator (Table I), nuclear genes controlled by this regulatory protein remain to be identified. The abundance of TAF-1 mRNA in roots suggests that this factor may regulate genes that are preferentially expressed in roots, e.g. alcohol dehydrogenase gene (DeLisle and Ferl, 1990). The role of TAF-1 in leaf is less clear. This factor can potentially interact with the G-box motif located upstream of *rbcS* and *Cab* genes (cf. Gilmartin *et al.*, 1990; Schindler and Cashmore, 1990). Because a motif I tetramer gives little or no expression in leaf, it appears unlikely that its cognate factor can function independently. On the other hand, a higher affinity binding site, e.g. PA, may overcome the problem of low factor abundance and confer leaf expression. We favor the hypothesis that TAF-1 interacts with other

factors to regulate the transcription of *rbcS* and *Cab*, and that this synergistic interaction is essential for high level expression of these photosynthetic genes in leaf. This may explain why mutation of the G-box motif reduces drastically the expression level of *Arabidopsis rbcS-1A* in transgenic tobacco plants (Donald and Cashmore, 1990). A similar situation may also apply to the *P.crispum chs* genes (Schulze-Lefert *et al.*, 1989a,b), where there is *in vivo* evidence for a requirement of the G-box-binding protein in UV-inducible expression.

Recently, Guiltinan *et al.* (1990) reported that a 75 bp fragment of the wheat Em gene can give ABA-responsive transcription in transient assays using rice protoplasts. This fragment contains two conserved G-box-like motifs, Em1a GGACACGTGGC and Em1b GCACACGTGCC, both of which have the CACGTG core sequence. Mutation of Em1a motif reduced the ABA induction ratio from 11 to 2 indicating that Em1a is necessary for the hormone induction. It is not known, however, whether Em1a alone can mediate ABA-responsive transcription or whether it has to interact with other *cis* elements within the 75 bp fragment for this activity. Guiltinan *et al.* (1990) isolated a wheat partial cDNA clone encoding a DNA-binding protein, designated EmBP-1, that binds to the Em1a motif, as well as the *hex* element of wheat histone H3 promoter (Table II). In the case of tobacco, we found that TAF-1 also binds to the *hex* element in addition to motif I (Figure 6). Neither motif I tetramer (Table I) nor the *hex* tetramer (E.Lam and N.-H.Chua, in preparation) can confer ABA-inducible expression on a GUS reporter gene in transgenic tobacco. By contrast, a *hex* mutant element that has greatly reduced affinity for TAF-1 (Figure 6) can confer ABA-responsive transcription in transgenic tobacco (E.Lam and N.-H.Chua, in preparation). These results would indicate that, at least in tobacco, motif I and TAF-1 are not directly involved in ABA-responsive gene expression. It is possible, however, that motif I may interact with different regulatory factors in rice as opposed to tobacco and functions as an ABRE in rice. Such differences between monocot and dicot transcription systems have been noted previously (Keith and Chua, 1986).

Materials and methods

Isolation of TAF-1 recombinant phage

A random-primed cDNA library was constructed in λ zap vectors using poly(A) RNA prepared from tobacco seedlings (*N.tabacum* cv. SR1) adapted in the dark for 2 days. The amplified library was screened with a labeled oligonucleotide fragment that spans between −275 and −206 of *Rab16B* (Figure 1A). We used essentially the screening protocol of Singh *et al.*, (1988) with minor modifications by Katagiri *et al.* (1989).

Nucleotide sequence analyses

Single-stranded templates were prepared from *E.coli* HB101 after infection with phage IR408 (Russell *et al.*, 1986). Nucleotide sequences of both strands were determined by a Sequenase TM sequencing kit (USB) using common primers and synthesized primers. Sequence data were analyzed by DNASIS and PROSIS programs (Hitachi) on an IBM PS12 computer.

Gel mobility-shift assays

Gel mobility-shift assays were performed according to Green *et al.* (1987). The assay mixture contained tobacco nuclear extract (7.5 μg protein) or *E.coli* extract (5 μg protein), 0.2 ng of binding probe (2×10^4 c.p.m.) and 5 μg of poly(dI−dC) in 5 μl of B buffer (20 mM HEPES−KOH, pH 7.5), 40 mM KCl, 1 mM EDTA, 10% glycerol and 0.5 mM DTT. Tobacco nuclear extract was prepared as described (Green *et al.*, 1987). *E.coli* cells containing recombinant plasmids were grown to early log phase and incubated with 2 mM IPTG for 4 h. Cells were collected and resuspended in buffer A (50 mM Tris−HCl, pH 7.5, 20% glycerol, 1 mM EDTA and 5 mM DTT). The suspension was sonicated and the homogenate centrifuged at 10 000 *g* for 15 min. The supernatant fraction was divided into aliquots, frozen in liquid nitrogen and stored at −80°C. Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer. Full-length products were purified on denaturating polyacrylamide gels, annealed and cloned into the *Hin*dIII−*Xho*I site of a pEMBL12$^+$ derivative (Dante *et al.*, 1983). Plasmid DNA containing the oligonucleotide insert was digested with *Hin*dIII and *Xho*I and labeled by fill-in reaction. The labeled insert was purified by polyacrylamide gel electrophoresis and used as binding probes.

Partial purification of recombinant TAF-1

To 10 ml of *E.coli* extract prepared as described above, 2.43 g of ammonium sulfate was gradually added over 30 min to obtain 40% saturation. Protein precipitation was collected by centrifugation at 15 000 *g* for 30 min and resuspended in 1.25 ml of buffer A and dialyzed against buffer A containing 20 mM NaCl for 4 h with three changes of 200 ml each. After dialysis, the extract was centrifuged in a microfuge for 10 min to remove insoluble materials. The supernatant fraction was divided into aliquots which were frozen in liquid nitrogen and stored at −80°C.

Northern and Southern analysis

Poly(A)RNA was prepared (Nagy *et al.*, 1988; Katagiri *et al.*, 1989), separated in formaldehyde gels and blotted onto Nitran filters. The filters were hybridized to the labeled *Eco*RI fragment (1.2 kb) of the cDNA clone 5a or the β-ATPase cDNA (Boutry and Chua, 1985) in a solution containing 6 × SSC, sonicated salmon testis DNA, 0.5% SDS, 0.2% Ficoll at 37°C for 24 h. Filters were washed in 0.1 × SSC at 65°C and autoradiographed. High molecular weight DNA was isolated from tobacco leaf (Ausubel *et al.*, 1987), and Southern blot analysis was performed as described by Maniatis *et al.* (1982).

Production of transgenic plants

Tetramers of wild type and mutant motif I (Figure 1A) were placed upstream of the vectors X-GUS-90 (Benfey *et al.*, 1989). *Agrobacterium tumefaciens* (GV3111SE) cells harboring the recombinant plasmid vectors were used to inoculate leaf discs of *N.tabacum* cv. SR1 and regenerated shoots were selected on medium containing kanamycin (200 μg/ml) (Horsch *et al.*, 1985). After rooting, transgenic plantlets were transferred to soil and grown to maturity in a greenhouse. R-0 plants were selfed and R-1 seeds and seedlings were used for experiments.

β-glucuronidase (GUS) enzyme assays

GUS enzyme activities in tobacco extracts were determined essentially as described (Jefferson *et al.*, 1987). Fluorescence was measured with a Perkin-Elmer LS5 fluorimeter. A solution of 100 mM 4-methyl umbelliferone (MU) in 0.2 M sodium carbonate was used to calibrate the fluorescence intensity. Histochemical staining of GUS activity was according to published protocols (Jefferson *et al.*, 1987; Benfey *et al.*, 1989).

High velocity microprojectile bombardment

Five μg plasmid DNA was coated onto tungsten powder and delivered to leaf sections by high velocity acceleration of the tungsten particles using a home-made instrument designed by Professor Konstantin Goulianos (Laboratory of Experimental Physics, The Rockefeller University). This instrument is based on the principle described by Klein *et al.* (1988, 1989). After bombardment, the leaf sections were incubated in a moist chamber in the dark at room temperature for 12 h before measurement of GUS activity.

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