Two G-Box-Related Sequences Confer Different Expression Patterns in Transgenic Tobacco

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We have analyzed the expression patterns conferred by two G-box-related motifs, a perfect palindromic sequence (PA, 5'-GCCACGTGGC-3') and motif I (Iwt, 5'-GTACGTGGCG-3'), in transgenic tobacco plants. A mutant version of motif I, Imu, was used as a negative control. PA is present in the promoters of several different genes, whereas Iwt is a conserved sequence found in abscisic acid-inducible promoters. Previously we have demonstrated that PA and Iwt, but not Imu, can bind to the tobacco transcription activator TAF-1 in vitro, with the PA sequence showing a 70-fold higher affinity as compared to Iwt. We found that tetramers of PA and Iwt, which differ by only 2 bp per 10-bp repeat, confer very different tissue-specific and expression patterns in transgenic tobacco plants. PA confers preferential expression in root tissues with a low level of activity in leaves, whereas Iwt directs developmentally regulated expression in seeds beginning 15 days after petals have fully expanded until seed maturation. Imu appears to be inactive because it gives the same expression pattern as the -90 cauliflower mosaic virus 35S promoter control. RNA gel blot analysis showed that the expression pattern of TAF-1 mRNA is similar to that directed by PA, suggesting that TAF-1 may be involved in the transcriptional regulation of PA.

INTRODUCTION

A central problem in eukaryotic molecular biology is to understand the mechanisms that regulate the expression of specific genes in a temporal or tissue-specific manner. Plants are particularly well suited to study this problem because transgenic plants can be rapidly and easily generated, and the expression of a convenient reporter gene can be monitored, thus allowing the functional analysis of promoter sequences in most cells throughout the plant's life cycle. Generally, it has been found that sequences located 5' of the coding regions of plant genes contain many of the transcriptional signals necessary for quantitative and qualitative (i.e., tissue-specific and/or developmentally regulated) control of gene expression. These sequences appear to control RNA polymerase activity by interacting with trans-acting factors. cis-Regulatory DNA sequences can be the target of more than one factor, which, in turn, could interact with different sequences with different affinity (Johnson and McKnight, 1989).

In plants, the G-box element exemplifies the ability of a certain DNA sequence to interact promiscuously with multiple binding proteins. This element was first identified as an 11-bp sequence (5'-C/A-ACACGTGGCA-3') located upstream of many genes encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) (Giuliano et al., 1988). Since then, regulatory sequences containing G-box or related motifs with the CACGTG hexanucleotide core have been identified in the promoters of a diverse set of unrelated genes, including those controlled by visible and UV light (Schultze-Lefert et al., 1989), abscisic acid (Guiltinan et al., 1990), wounding (Rosahl et al., 1986), or anaerobiosis (DeLisle and Ferl, 1990). Recent data indicate that plant nuclear extracts contain a number of binding activities with specificity for G-box and related sequences (Staiger et al., 1991; Williams et al., 1992) and, indeed, several cDNA clones encoding proteins that specifically interact with sequences containing the CACGTG core have been isolated and include wheat Em binding protein EmBP-1 (Guiltinan et al., 1990), wheat histone binding protein HBF-1a (Tabata et al., 1991), tobacco transcription activator TAF-1 (Oeda et al., 1991), parsley common plant regulatory factors CPRF-1, CPRF-2, and CPRF-3 (Weisshaar et al., 1991), and Arabidopsis G-box binding factors GBF-1, GBF-2, and GBF-3 (Schindler et al., 1992). Interestingly, all these proteins belong to the basic/leucine zipper (bZIP) class of transcription factors, i.e., they possess a basic domain abutting a leucine repeat. It has been shown that bZIP proteins bind to their target sites as a dimer (cf. Johnson and McKnight, 1989).

The TAF-1 cDNA clone was isolated by screening a tobacco cDNA expression library for proteins that show affinity for motif I (Iwt, 5'-GTACGTGGCG-3'), a conserved sequence found in the promoters of different abscisic acid-responsive genes (see Skriver and Mundy, 1990 for a review). Because TAF-1 is a bZIP...
protein and because lwj contains a G-box–related core sequence TACGTG, we reasoned that the preferred binding site of TAF-1 may be the perfect palindrome (PA, 5′-GCCACG-TGCC-3′), which contains the G-box hexameric core sequence CACGTG. This hypothesis was subsequently confirmed by gel shift assays, and we found that TAF-1 binds to PA with a higher affinity (≈70 times) than lwj (Oeda et al., 1991).

The finding that TAF-1 can bind to two related sequences in vitro, PA and lwj, although with different affinity, poses interesting questions with regard to the interaction of these sequence elements with the transcription activator in vivo. RNA gel blot analysis demonstrated that TAF-1 is highly expressed in roots but poorly expressed in leaves and stems (Oeda et al., 1991). In the simplest case, if both elements interact with TAF-1 in vivo, then they should confer an expression pattern similar to that exhibited by TAF-1; however, their activities in the expressing tissues may differ quantitatively, reflecting in some way their different binding affinity for the factor. Alternatively, if the elements interact with different factors in vivo, then different expression profiles should ensue. Other more complicated scenarios are also possible.

In an attempt to elucidate the functional properties of lwj and PA in vivo, to shed some light on the TAF-1 function, we made transgenic tobacco plants containing lwj or PA tetramers linked to a β-glucuronidase (GUS) reporter gene. GUS activity in different tissues and at different stages of plant development was analyzed quantitatively and qualitatively. Furthermore, we examined the expression pattern of TAF-1 mRNA in different tissues of tobacco plants. Here, we show that the two closely related sequences PA and lwj, which differ only in 2 bp per 10-bp repeat, confer dramatically different expression patterns in transgenic tobacco plants: lwj promotes expression in developing and mature seeds, whereas PA confers preferential expression in roots. We also show that TAF-1 mRNA is not expressed in seeds, although it is highly expressed in roots. On the basis of these results, possible interactions between TAF-1 and PA and lwj sequences for regulating gene expression are discussed.

RESULTS

Constructs

To investigate the functional properties of PA and motif I (lwj), we synthesized head-to-tail tetramers comprising tandem copies of these sequences, and inserted them upstream of the truncated cauliflower mosaic virus (CaMV) 35S promoter in the X-GUS-90 vector (construct 1) to generate constructs 2 and 3, respectively. The X-GUS-90 vector contains the −90 to +8 (A domain) region of the CaMV 35S promoter fused to the GUS coding sequence, with a 3′ fragment of the pea rbcS-3C gene (Benfey and Chua, 1989). As a negative control, we also synthesized a tetramer of a motif I mutant, TGACTGTCTC, designated as Imu, previously shown to have no binding activity for TAF-1 (Oeda et al., 1991). The Imu tetramer was also inserted upstream of the truncated CaMV 35S promoter in the X-GUS-90 vector to give construct 4. Figure 1A shows the structures of these chimeric constructs, and Figure 1B shows the comparison of the different tetramer sequences. The chimeric constructs were transferred to Agrobacterium and tobacco leaf discs were transformed as described in Methods. The expression of the GUS reporter gene was analyzed in primary transformants as well as in their progeny at different developmental stages.

Expression in Mature Plants

We first measured quantitatively GUS activity in leaves and roots from at least seven independent transgenic plants for each construct. Figure 2A shows that leaves from plants carrying constructs 3 and 4 expressed a similar level of GUS activity as those from plants containing the X-GUS-90 vector alone (construct 1). By contrast, GUS activity in leaves harboring construct 2 was three to five times higher. The value of GUS activity in the leaves was not affected by the growth conditions of the plants (soil or tissue culture grown plants). Figure 2B shows the expression levels in root tissue. Constructs 1 and 4 gave similar GUS expression levels, whereas constructs 2 and 3 were >-10 and two times higher, respectively. We concluded from these results that, in mature plants, PA can confer a high level of expression in roots and, to a lesser extent, Iwt contains a G-box–related core sequence TACGTG, we reasoned that the preferred binding site of TAF-1 may be the perfect palindrome (PA, 5′-GCCACG-TGCC-3′), which contains the G-box hexameric core sequence CACGTG. This hypothesis was subsequently confirmed by gel shift assays, and we found that TAF-1 binds to PA with a higher affinity (≈70 times) than lwj (Oeda et al., 1991).
Figure 2. GUS Activities of Transgenic Tobacco Plants Carrying the Various Chimeric Constructs.

Chimeric constructs are as described in Figure 1. GUS activities were measured as described in Methods. Data from seven to 10 independent transgenic plants for each construct are shown; the highest and the lowest values are not represented. Activity, represented in logarithmic scale, is expressed as picomoles of 4-methylumbelliferone produced per minute per milligram of protein. Each dot represents the activity of an independent transgenic line.
(A) Leaves from 7- to 10-week-old plants grown in a greenhouse and from 7-week-old plants grown on supplemented MS medium.
(B) Roots from 7-week-old plants grown on supplemented MS media.
(C) Mature seeds.

degree, in leaves. On the contrary, Iwt and Imu appear to be relatively inactive in these tissues. The fivefold to 10-fold higher level of GUS activity conferred by construct 1 (X-GUS-90 vector) in roots as compared to leaves confirms our previous observation that domain A (−90 to +8) of the CaMV 35S promoter shows preferential expression in roots (Benfey et al., 1989). Because constructs 2, 3, and 4 were all made in the context of the −90 CaMV 35S promoter, it is possible that the expression patterns obtained with PA reflect the interaction of this sequence with domain A.

Expression during Seed Development

R1 seeds from transgenic plants containing constructs 1 through 4 were analyzed at several stages of development. For this purpose, seeds were harvested at 10, 15, and 20 days after petals had fully expanded (DAF), and also at maturity. Figures 3A to 3L show the GUS expression pattern in transgenic seeds. The seeds were sectioned, stained for GUS activity, and examined by light microscopy as described in Methods. In the case of mature seeds, the GUS activity in soluble extracts was also determined quantitatively (Figure 2C).

Seeds that contained PA or Imu did not show any specific GUS staining when compared to control seeds containing the X-GUS-90 vector alone (cf. Figures 3A to 3C and Figures 3D to 3F). In all three cases, GUS activity was localized, beginning at 15 DAF (data not shown), in the radicle of the embryo and in the endosperm cells at the radicle pole (Figures 3A to 3F). By contrast, Iwt conferred regulated expression in developing seeds. GUS expression was first detected in embryos from seeds at 15 DAF (Figures 3G to 3I). The expression progressively increased until seed maturity, when GUS activity was present in the entire embryo as well as the endosperm (Figures 3J to 3L). The different seed expression patterns conferred by PA or Imu on one hand, and Iwt on the other, were also reflected in quantitative GUS assay of extracts prepared from mature seeds (Figure 2C). Seeds with Iwt showed ~10 times more GUS activity than seeds with PA or Imu, which gave the same level of activity as seeds from control plants (construct 1).

These results indicate that whereas PA and Imu are inactive in seeds, Iwt can confer specific expression in the embryo and endosperm. Moreover, this expression appears to be developmentally regulated during seed development because GUS activity appears at 15 DAF and subsequently increases until seed maturity. GUS expression in the radicle of the
Figure 3. Histochemical Localization of GUS Expression in Seeds from Representative Transgenic Plants Containing PA, lwt, and Imu Sequences.

(A) to (C) Mature seeds containing PA. Similar results were obtained with mature seeds containing Imu tetramers.

(D) to (F) Mature seeds containing the X-GUS-90 vector.

(G) to (I) Capsules of seeds containing lwt tetramers at 10, 15, and 20 DAF, respectively.

(J) to (L) Mature seeds containing lwt tetramers.

(A), (D), and (J) show whole seeds; (B), (E), and (K), embryos; (C), (F), and (L), endosperms. C, cotyledon; En, endosperm; Em, embryo; Ra, radicle; Rp, radicle pole of endosperm; Cp, cotyledon pole of the endosperm. Arrows in (H) and (I) indicate GUS activity detected in developing seeds.
Expression Patterns Conferred by G-Box-Like Motifs

embryos, and in the endosperm cells at the radicle pole, was likely due to the −90 to +8 CaMV 35S promoter fragment (A domain) from the X-GUS-90 vector, as already described (Benfey et al., 1989).

Expression in Young Seedlings

R1 seeds from transgenic plants containing constructs 1 through 4 were sterilized and germinated on media as described in Methods. Seedlings were removed at 7 days (stage of two cotyledons) and 15 days (stage of two cotyledons and first two leaves), and then processed for detection of GUS activity at the cellular level.

Figure 4 shows that 7-day-old seedlings containing PA demonstrated strong GUS activity in roots and weak GUS activity in cotyledons (Figure 4A). At 15 days, seedlings containing PA showed very strong staining in roots, and intermediate staining in the cotyledons and in the first two leaves (Figure 4B). As a negative control, seedlings containing Imu demonstrated an identical expression pattern to that of X-GUS-90 control seedlings, i.e., high GUS activity was only detected at the root tips in 7-day-old as well as 15-day-old seedlings (Figures 4C and 4D). In seedlings containing Iwt, strong GUS activity was evident in the cotyledons, as well as at the root tips (Figure 4E). No GUS activity was visible in the first two leaves (Figure 4F).

From these results, we concluded that PA and Iwt confer tissue-specific expression in seedlings and, moreover, this expression is developmentally regulated during seed germination. In the case of PA, expression is very strong in roots and, to a lesser degree, in cotyledons and leaves. It should be stressed that this pattern of expression appears only after seed germination. On the other hand, in young seedlings containing the Iwt sequences, expression is detected in cotyledons but, interestingly, not in leaves. In all of the four constructs, the expression detected in the root tip can be attributed to the CaMV 35S A domain in the X-GUS-90 vector (Benfey et al., 1989).

Expression Pattern of TAF-1 mRNA

We have previously characterized a cDNA clone encoding a tobacco transcription activator designated as TAF-1 (Oeda et al., 1991). TAF-1 is a bZIP protein that binds to both Iwt and PA, albeit with strikingly different affinity, but not to Imu. Because the expression patterns conferred by cis-regulatory elements may reflect the abundance of the transcription factors

Figure 4. Histochemical Localization of GUS Expression in Seedlings from Representative Transgenic Plants Containing PA, Iwt, and Imu Sequences.

(A) and (B) Seedlings containing PA tetramers.
(C) and (D) Seedlings containing Imu tetramers. Similar results were obtained with seedlings containing the X-GUS-90 vector alone.
(E) and (F) Seedlings containing Iwt tetramers.
(A), (C), and (E) show 7-day-old seedlings; (B), (D), and (F), 15-day-old seedlings. C, cotyledon; R, root; Rc, root cap; L, first leaves.
that interact with them, we compared the expression pattern of TAF-1 mRNA to those conferred by PA and Iwt.

In a previous report, we showed that TAF-1 mRNA is 10 to 20 times more abundant in roots than in leaves and stems (Oeda et al., 1991). The transcriptional activity of Iwt in tobacco seeds (Figures 2 and 3) prompted us to analyze the expression of TAF-1 mRNA in this organ. We chose to investigate the TAF-1 mRNA level in developing seeds at 20 DAF because at this stage of seed development, Iwt already conferred a high GUS expression level (see above). Figure 5, top panel, shows that TAF-1 mRNA was not detected in developing seeds (20 DAF), nor in leaves. A longer exposure of the autoradiogram only revealed a faint band corresponding in size to that of the TAF-1 mRNA in leaves (data not shown). As an internal control for the RNA gel blot analysis, the mRNA coding for the constitutively expressed mitochondrial β-ATPase gene (Boutry and Chua, 1985) was present at a higher level in seeds as opposed to leaves and roots (Figure 5, bottom panel). These results suggest that the expression pattern of TAF-1 mRNA is similar to that conferred by PA (see above).

Among the three GBFs recently cloned from Arabidopsis, GBF-3, which shares sequence homology with TAF-1, is also highly expressed in roots with little expression in leaves (Schindler et al., 1992). It would be interesting to see whether GBF-3 is also poorly expressed in seeds, as is the case with TAF-1 reported here.

DISCUSSION

We have previously reported that the tobacco transcriptional activator TAF-1 is able to bind to two G-box-like sequences, TAF-1 and β-ATPase. In this study, we compared the in vivo expression patterns conferred by these two short sequences. As a negative control, we also investigated the activity of Imu, a motif I mutant, which is unable to bind to the activator in vitro (Oeda et al., 1991). Our results show that PA and Iwt tetramers, which differ only in 2 bp per 10-bp repeat (Figure 1B), confer strikingly different expression patterns in transgenic tobacco plants. The Imu tetramer, on the other hand, appears to be inactive as a cis-regulatory element because it has no noticeable effect on the expression of the −90 CaMV 35S promoter. Figure 6 summarizes in a schematic form the expression patterns obtained with constructs 1 to 4 at different stages of plant development.

PA confers high-level expression in roots, low-level expression in leaves, and little or no expression in seeds (Figures 3A to 3C and 4A and 4B). Iwt, on the other hand, confers preferential expression in seeds but in a developmentally regulated manner. GUS activity appears only at 15 DAF and increases steadily thereafter (Figures 3G to 3I), and in mature seeds the activity is detected in the entire embryo as well as the endosperm. Although some GUS activity is found in cotyledons of germinating seedlings (Figures 4E and 4F), this could be due to residual GUS enzyme synthesized during seed development.

The contrasting and almost nonoverlapping expression profiles conferred by PA and Iwt strongly suggest that these two cis-regulatory elements interact with different trans-activating
factors in vivo. We favor the hypothesis that the transcription activator TAF-1 is the cognate factor for PA for the following two reasons: (1) PA is a high-affinity binding site for the activator (Oeda et al., 1991), and (2) the expression pattern conferred by PA parallels that of TAF-1. Previous bombardment experiments using a 3SS-TAF-1 chimeric construct demonstrated that a transient increase in the expression level of TAF-1 can activate an Iwt-linked transgene in tobacco leaves (Oeda et al., 1991). Notwithstanding this observation, we show here that Iwt is inactive in roots even though this organ expresses TAF-1 at a high level. This result suggests that the high concentration of TAF-1 in roots is still unable to compensate for the low binding affinity of Iwt for this factor.

In comparison to other known seed-specific promoters, the expression pattern conferred by Iwt is of particular interest because it appears to direct expression in both embryos and endosperm. The cis-acting elements studied thus far from seed-specific genes promote expression in specific regions of the seed (cf. Goldberg et al., 1989 for a review; Bustos et al., 1991). Only hex-3 (5'-GGACGCGTGGC-3'), a mutant derivative of the hex motif located in the wheat histone H3 promoter (Tabata et al., 1989), has been shown to confer activity in the embryo as well as in the endosperm of tobacco seeds (Lam and Chua, 1991). Whether Iwt and hex-3 interact with the same or different factors remains to be determined. We note that Iwt (GTACGTGGC) shares striking sequence homology (1 of 9 bp are identical) to the Opaque-2 target binding site (TCT-ACGTGGC) in the 5' region of the 22-kD zein gene that is expressed predominantly in maize endosperm (Schmidt et al., 1992).

An important consideration for the interpretation of our expression studies is that the constructs were made in the context of the -90 CaMV 35S promoter (Figure 1A). As described previously, this promoter confers expression in the radicle of the embryo, the radicle pole of the endosperm, and the tips of young and mature roots (Benfey et al., 1989). By contrast, construct 2, which contains the PA tetramer, confers strong expression in the entire root including root hairs (Figures 4A and 4B). In addition, the GUS activity in roots containing construct 2 is ~10 times higher than in those containing the -90 CaMV 35S promoter alone (Figure 2). We have shown previously that a 21-bp element, designated as-as1, is responsible for the root expression of the -90 CaMV 35S promoter (Lam et al., 1989). It is possible that the strong expression in root as well as the weak expression in leaf, obtained with construct 2, are generated by a synergistic interaction between the PA tetramer and the as-1 element. Similar consideration may also apply to the preferential seed expression seen with construct 3.

In this study, we present direct evidence that two closely related plant regulatory sequences such as PA and Iwt can confer completely different expression patterns in transgenic plants. Clearly, these two sequences can be used to direct tissue-specific and developmentally regulated expression of target genes for basic research as well as for biotechnological applications.

METHODS

Constructs

Construct 1 contains the cauliflower mosaic virus (CaMV) 35S promoter fragment from -90 to +8 (A domain) fused to the β-glucuronidase (GUS) coding sequence (Jefferson et al., 1987). The 3' end from the pea rbcS-3C gene was placed downstream of the GUS sequence to provide for a polyadenylation signal. This construct was inserted into pMON505 to give the X-GUS-90 vector, which has been described in detail elsewhere (Benfey and Chua, 1989). For construct 2, a head-to-tail tetramer of the perfect palindromic sequence (PA) 5'-GCCACGTGGC-3' was synthesized with a HindIII site at the 5' end and a XhoI site at the 3' end. The tetramer was then cloned between the HindIII and XhoI sites into the pEMBL12 derivative sequenced and then inserted at -90 of X-GUS-90. The same strategy was used to generate constructs 3 and 4 except that the tetromers synthesized corresponded to the wild-type motif I (Iwt) 5'-GGTACGTGGC-3' and the mutant motif I derivative (Imu) 5'-TGACTGTGGC-3', respectively.

Transgenic Plants

Constructs were mobilized into Agrobacterium tumefaciens GV3111SE, and tobacco plants (Nicotiana tabacum cv SR1) were used for transformation. Shoots were regenerated on medium containing 200 μg/mL kanamycin (Rogers et al., 1986). After rooting, transgenic plantlets were transferred to soil and grown in a growth chamber, or maintained, by cuttings, in Plantcons containing MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.7% agar, 100 μg/mL kanamycin, and 500 μg/mL carbenicillin. Primary transformants were allowed to self-fertilize and seeds were collected, sterilized, and germinated on supplemented MS media (see above). Seedlings were maintained at 26°C under 16-hr light and 8-hr dark conditions. Seven to 10 independent transgenic plants were analyzed for each construct.

Histochemical Staining

GUS histochemical staining was carried out essentially as described by Jefferson et al. (1987). The development stages of the seeds used for the assays were determined by tagging flowers of primary transformants when petals had fully expanded (0 DAF). At various intervals hereafter (10, 15, and 20 DAF), capsules were removed and 200-μm sections were prepared with a cryotome. Sections were stained by placing them directly into a histochemical substrate solution containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronidase (X-gluc) and 50 mM sodium phosphate buffer, pH 7.0, on a microscope slide for 12 hr in a humidified chamber at 37°C. Mature seeds were stained as described by Benfey and Chua (1989). To rule out possible diffusion of the GUS enzyme or dye from one tissue to another during incubation, embryos were removed from endosperms prior to incubation with the substrate. Seven- and 15-day-old seedlings were removed from the Petri dishes, placed directly into the X-gluc solution, and incubated as described for capsule sections (see above). After the incubation, chlorophyll was cleared as described previously (Benfey and Chua, 1989).

GUS Enzyme Assays

GUS enzyme assays were performed mainly as described by Jefferson et al. (1987). Extracts were made from leaves and roots of 7-week-old
plants grown on supplemented MS media (see above), from leaves of 7- to 10-week-old plants grown in a greenhouse, and from mature seeds. Ten micrograms of protein were incubated with 4-methylumbelliferyl glucuronide solution for 60 min at 37°C. The reactions were stopped by adding 2.5 mL of 0.2 M sodium carbonate, and fluorescence was measured with a Perkin-Elmer LS5 fluorometer by using a solution of 100 nM 4-methylumbelliferone in 0.2 M sodium carbonate for calibration.

**RNA Gel Blot Analysis**

Poly(A) RNA was extracted from seeds at 20 DAF, and from leaves and roots of 7-week-old transgenic tobacco plants grown on supplemented media (see above). RNA was electrophoresed in glyoxal gels and blotted according to standard protocols. Filters were hybridized to the 1.2-kb-labeled EcoRl fragment of TAF-1 cDNA (Oeda et al., 1991), or to the β-ATPase cDNA (Boutry and Chua, 1985) in a solution of 50% formamide, 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5 x Denhardt’s solution (1 x Denhardt’s solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA) at 42°C for 20 hr. Filters were washed in 1 x SSC, 0.5% SDS at 65°C for 1 hr and autoradiographed.

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