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Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development

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The cauliflower mosaic virus (CaMV) 35S enhancer is able to confer strong constitutive expression in plants. We have previously defined two domains within this enhancer that can confer different tissue-specific expression patterns throughout development. We show here that the upstream domain (B) has a modular organization. It contains at least five subdomains that are able to confer distinct expression patterns when fused to the downstream domain (A). When fused to a minimal promoter only three of the five subdomains give any expression in the early stages of plant development. Comparison of the expression patterns conferred by the subdomains alone, in combination with the downstream domain or in combination with other subdomains provides evidence for synergistic interactions among *cis*-elements within the 35S enhancer.

Key words: *cis*-elements/developmental regulation/GUS reporter gene/histochemical localization/transgenic plants

Introduction

The 35S promoter of cauliflower mosaic virus (CaMV) acts as a strong constitutive promoter in most organs of transgenic plants (Odell *et al.*, 1985; Jensen *et al.*, 1986; Jefferson *et al.*, 1987; Kay *et al.*, 1987; Sanders *et al.*, 1987). The upstream region from −343 to −46 has been shown to function in an orientation and distance independent manner (Kay *et al.*, 1987; Nagy *et al.*, 1987; Fang *et al.*, 1989). We are studying this viral enhancer as a model system to characterize the modular organization of enhancers that function in higher plants. We have previously defined two domains within this enhancer, each of which is able to confer tissue-specific and developmentally regulated expression in transgenic plants (Benfey *et al.*, 1989). The downstream domain (−90 to +8) termed domain A (which also contains the TATA box at −31 to −25, see Figure 1A) is able to confer expression principally in root tissue (Benfey *et al.*, 1989). A *cis*-element located between −85 and −64 appears to be primarily responsible for this expression. The element, termed 'activation sequence' (as)-1, contains a tandem repeat of the sequence, TGACG (Figure 1A) and binds a nuclear factor, 'activation sequence factor' (ASF)-1, in tobacco nuclear extracts (Lam *et al.*, 1989). Mutation of the TGACG motifs within the intact promoter causes a decrease in expression in root (Lam *et al.*, 1989). Insertion of as-1 into a promoter that normally expresses only in photosynthetic tissue results in augmented expression in root (Lam *et al.*, 1989). We have isolated a cDNA clone for a factor that binds to this motif (Katagiri *et al.*, 1989). The level of the RNA that hybridizes to this cDNA is 5- to 10-fold higher in root than in leaf tissue (Katagiri *et al.*, 1989). The preferential root expression conferred by domain A can thus be attributed to the higher level of a factor in root that interacts with the as-1 element.

It should be emphasized, however, that expression conferred by domain A is not exclusively in root (Benfey *et al.*, 1989) nor is the nuclear factor, ASF-1, found exclusively in extracts from root (Katagiri *et al.*, 1989; Lam *et al.*, 1989). Previous work has suggested a more complex role for this domain than merely providing root expression to the 35S promoter. Domain A has been shown to interact synergistically with other enhancer sequences to activate transcription in leaf (Poulson and Chua, 1988; Fang *et al.*, 1989; Lam *et al.*, 1989).

In contrast to domain A, much less is known about the upstream domain (−343 to −90), termed domain B. This domain is almost entirely within an open reading frame (ORF) that codes for a protein implicated in host range specificity and disease symptoms (Schoelz *et al.*, 1986; Baughman *et al.*, 1989) (the stop codon of the ORF is at position −99, Figure 1A). Although domain B is able to confer expression in most cell types of leaf and stem as well as in vascular tissue of the root (Benfey *et al.*, 1989) it is not known whether one or several *cis*-elements residing within domain B are responsible for this expression profile. Here we show that this domain has a modular organization. We divided domain B into five subdomains, each of which is able to confer a different pattern of expression. In the early stages of plant development only three of the subdomains confer detectable levels of expression when fused to a minimal promoter. When placed upstream of domain A, however, all five subdomains give expression patterns that differ from that of domain A alone. Comparison of the expression pattern of the entire domain B and a combination of two subdomains provides further evidence for synergistic interactions among *cis*-elements found in the 35S enhancer.

Results

Experimental design

Previous analysis of the 35S promoter showed that deletion of four regions caused reductions in transcriptional activity in mature leaf tissue (Fang *et al.*, 1989). Based on these results we divided domain B (−343 to −90) into five subdomains at the sites used in the previous study as well as at one additional site within the most 5′ region (Figure 1A). The subdomains were numbered B1 through B5 as indicated in Figure 1B. We will refer to the region from −90 to −46 as subdomain A1 (Figure 1B). The subdomains were inserted as head to tail tetramers into an expression vector that contained the TATA region of the 35S promoter

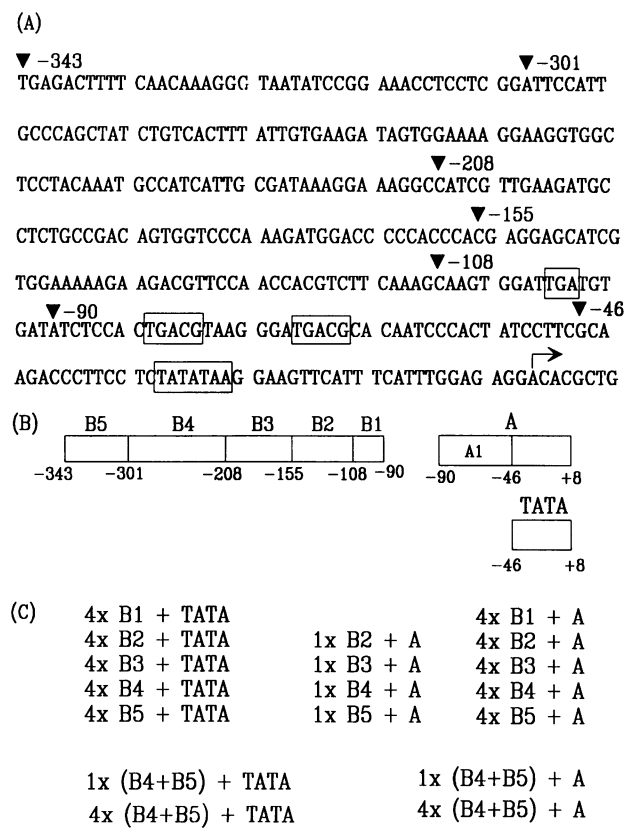

Fig. 1. Constructs with subdomains of the 35S enhancer. (**A**) The sequence of the 35S promoter from −343 to +8 with the breakpoints of the subdomains indicated with filled triangles. In boxes are the stop codon (TGA) for ORF VI at position −99, the two TGACG motifs and the TATA sequence. The start site of transcription is equivalent to map position 7435 of CaMV (Hohn *et al.*, 1982). (**B**) Schematic representation of the B subdomains and the two vectors. The difference between the A domain vector and the TATA vector is referred to as subdomain A1. (**C**) The subdomains were inserted as tetramers (4×) or monomers (1×) upstream of the A domain vector (+A) or as tetramers upstream of the TATA vector (+TATA). The combination of subdomains, B4+B5, is a fragment from −343 to −208 which was inserted as a tetramer and as a monomer upstream of both vectors. Control constructs analyzed include domain B inserted upstream of domain A and domain B inserted upstream of the TATA vector. Expression from the TATA vector alone was also analyzed. All constructs contained a transcriptional fusion to the GUS coding sequence.

(−46 to +8) fused to the *Escherichia coli* β-glucuronidase (GUS) coding sequence (Jefferson *et al.*, 1987) (Figure 1B and C). In order to analyze the interactions of the B subdomains with domain A the subdomains were inserted upstream of domain A (−90 to +8) fused to the GUS coding sequence (Figure 1B and C). Both tetramers and monomers of the subdomains (except for subdomain B1) were combined with domain A to determine the effect of multimerization on expression. A combination of subdomains B4 and B5 (−343 to −208) was also inserted as a monomer and a tetramer upstream of the TATA and the A domain vectors (Figure 1C). Since the B2 subdomain appeared to interact with domain A only as a tetramer, we inserted the tetramer in both vectors in the normal and in the reverse orientation to further characterize its ability to confer expression. Except for analysis of the B2 subdomain with a −72 to +8 minimal promoter vector, the B subdomains were not analyzed as monomers with the TATA vector.

Control constructs include the TATA vector alone and domain B fused to the TATA vector. Expression conferred by domain A alone and expression conferred by domain B fused to domain A have been described (Benfey *et al.*, 1989). The constructs were transferred into tobacco and expression of the GUS enzyme was assayed by histochemical localization at different developmental stages.

Expression in seed

R1 seeds from the primary transformants were harvested, sectioned and stained as described previously (Benfey *et al.*, 1989). At this stage of development only two subdomains confer detectable expression when inserted in the TATA vector. Subdomain B2 (4×B2) gives expression that is restricted to the cells at the tip of the radicle and to a small region in the endosperm tissue at the radicle pole (Figure 2B). Two distinct staining patterns were observed from subdomain B3 (4×B3). In four plants, subdomain B3 confers expression in a broad region of endosperm tissue at the radicle pole and in a narrow region of the radicle tip of the embryo (Figure 2C). In three other plants, expression in the cotyledons of the embryo and in the endosperm tissue adjacent to the cotyledons similar to that of the intact B domain was observed (data not shown).

In nearly all plants with B subdomains fused to domain A we observed staining in the radicle and in the endosperm tissue of the radicle pole. This is the expression pattern observed when domain A alone is present (Figure 2K). Plants containing subdomain B5 fused to domain A, 4×B5+A, exhibit only this expression pattern, indicating that this subdomain does not confer any additional expression at this stage of development (Figure 2J). For the other subdomains fused to domain A we detected some degree of expression in the cotyledons as well as in the radicle. 4×B1+A gives quite strong expression in the cotyledons (Figure 2F). 4×B2+A occasionally confers weak expression in the cotyledons but more commonly shows only very strong expression in the radicle (Figure 2G). 4×B3+A confers strong expression throughout the embryo and endosperm (Figure 2H). 4×B4+A gives expression in the tip of the cotyledons and appears also to express in the embryo axis (Figure 2I).

The expression pattern of the entire domain B fused to the TATA vector is identical to that described for domain B fused to the −72 to +8 promoter (Benfey *et al.*, 1989). Expression is strong in the cotyledons and is detected in the endosperm cells adjacent to the cotyledons (Figure 2L). Weak expression at the tip of the radicle is also observed in some plants. In plants containing the combination of subdomains as a monomer (B4+B5) occasionally weak expression in the cotyledons was observed (Figure 2M). As a tetramer, B4+B5 reproducibly confers expression in the cotyledons (Figure 2N). When fused to the A domain, (B4+B5)+A, expression is consistently observed in the cotyledons (Figure 2O). There was variation in the degree of staining in the radicle with this construct, from very weak staining (Figure 2O) to moderately strong staining. No staining was observed in seeds from 11 plants containing the TATA vector alone.

At this stage of development we find that the combination of two subdomains (B4+B5), which individually give no expression, confers a detectable pattern of expression. There are two possible explanations for these observations. Either

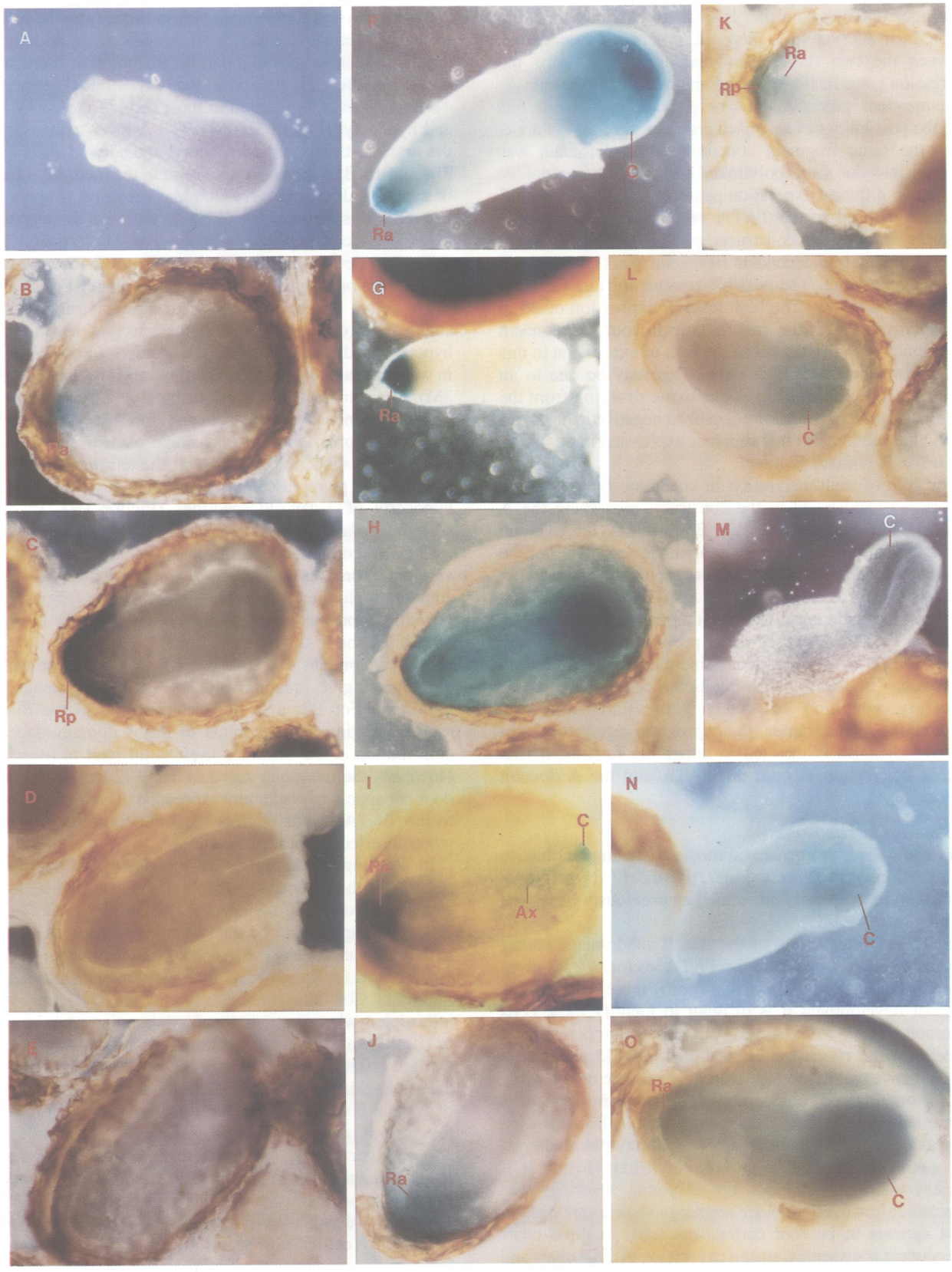

Fig. 2. Histochemical localization of expression in seeds of representative plants containing subdomain constructs. (In Figures 2 and 3 the first two columns of sections follow the same order.) (**A**) 4×B1; (**B**) 4×B2; (**C**) 4×B3; (**D**) 4×B4; (**E**) 4×B5; (**F**) 4×B1+A; (**G**) 4×B2+A; (**H**) 4×B3+A; (**I**) 4×B4+A; (**J**) 4×B5+A; (**K**) A domain; (**L**) B domain; (**M**) B4+B5; (**N**) 4×(B4+B5); (**O**) 4×(B4+B5)+A. Abbreviations: Ax, embryonic axis; C, cotyledons; Ra, radicle; Rp, radicle pole of the endosperm.

a *cis*-element that is important for this expression pattern was interrupted when the individual subdomains were isolated, or alternatively, there are synergistic interactions between the two subdomains that are essential for the expression pattern. For the subdomains fused to domain A, there are clear indications of synergistic interactions. In this case the possibility of interrupted *cis*-elements does not arise, since the same fragment containing the subdomain was inserted into the same polylinker sites in both vectors.

The two different expression patterns observed for the B3 subdomain fused to the TATA vector represent the only example among our constructs of qualitatively different patterns arising in independent transgenic plants that contain the same construct. The possible reasons for this variation are discussed below. The high level of expression of B3 in endosperm tissue at the radicle pole in four independent transgenics is surprising given the lack of expression in this tissue from the intact B domain. This may be due to an enhancement of expression in this tissue resulting from the multimerization of the subdomain or to negative interactions among the subdomains that suppress expression in this tissue when the B domain is intact.

The role of multimerization in changing the expression pattern was addressed in the constructs containing the A domain. For subdomains B3, B4 and B5 there is no qualitative difference in expression with the monomer or the tetramer. Subdomain B2, however, seems to enhance root expression only as a tetramer. We analyzed a construct that contained this subdomain fused as a monomer to the minimal promoter (−72 to +8) used in our previous analysis (Benfey *et al.*, 1989). In the seeds from nine plants we detected no expression indicating that multimerization may be a necessary condition to detect expression from this subdomain. As a tetramer, the expression pattern does not change when B2 is inserted in either the forward or the reverse orientation in both the TATA and the A domain vectors.

Expression in seedlings

Transgenic seedlings grown on media containing antibiotics for selection were harvested at 7, 10 and 17 days. Whole mounts were prepared and stained as previously described (Benfey *et al.*, 1989).

In R1 seedlings, three of the five B subdomains confer expression when inserted in the TATA vector. 4×B2 gives expression in the very tip of the root (Figure 3B). At this stage of development it is difficult to identify these cells accurately, but it would appear that expression is restricted to cells in the root cap. In seedlings, the B3 subdomain (4×B3) gave two expression patterns that were qualitatively similar but differed considerably in the intensity and extent of the staining. In four independent plants, B3 conferred expression in the cotyledons in 7- and 10-day-old seedlings, that was strongest at the base of the cotyledons (Figure 3C). No expression in the root tip of young seedlings was detectable; however, in older seedlings some expression in what appears to be root cortex was seen. In three other independent transgenics, strong expression in cotyledons and in root cortex was detectable even in young seedlings (data not shown). Expression from 4×B4 was only observed in one plant at the 17-day stage in the vascular tissue at the shoot apex (Figure 3D). No expression was detected in seedlings at any stage with subdomains 4×B1 (Figure 3A) or 4×B5 (Figure 3E).

When fused to the A domain, four of the five subdomains confer expression patterns that clearly differ from the pattern of the isolated subdomain or of domain A. In addition to the expression in root that can be attributed to domain A, 4×B1+A gives expression in the cotyledons (Figure 3F), 4×B3+A gives expression in most cells of the cotyledons and hypocotyl (Figure 3H), 4×B4+A gives expression in the vascular tissue of the hypocotyl and cotyledons (Figure 3I), and 4×B5+A gives expression in the mesophyll tissue of the cotyledons and leaves in older seedlings (Figure 3J). For the fifth subdomain, 4×B2+A, although there appears to be enhanced expression in the root (Figure 3G) it is difficult to determine whether this differs significantly from the expression conferred by domain A alone (Figure 3K).

The intact B domain confers strong expression in most cells of the cotyledons and in the vascular tissue of the hypocotyl and root (Figure 3L). In addition, some expression in what appears to be cells of the root cap is detected (Figure 3M). The combination of subdomains B4+B5 as a monomer gives an identical pattern of expression to that of B4. We observed expression only in a single plant in the vascular tissue of the shoot apex. However, the tetramer of B4+B5 reproducibly gives vascular expression in the cotyledons and hypocotyl of 7-day-old seedlings (Figure 3N). In older seedlings, vascular expression is also detected in root. B4+B5 in combination with the A domain confers strong vascular expression in hypocotyl and cotyledons as well as some mesophyll expression in the cotyledons (Figure 3O). No expression was detected in the seedlings from 11 plants containing the TATA vector alone.

For subdomains B3, B4 and B5, fused to domain A, monomers give qualitatively the same expression as tetramers. For B2, it is impossible to assess the role of multimerization in the context of the A domain since the contribution of the tetramer itself is difficult to ascertain. However, the monomer fused to the −72 to +8 promoter does not appear to confer any expression. The tetramer fused to the TATA vector confers the same expression pattern when inserted in either orientation.

In seedlings it appears that the detectable expression patterns of the isolated B subdomains do not add up to the expression pattern of the intact B domain. In particular, no subdomain appears to be able to confer strong expression in the vascular tissue of the hypocotyl. There are two possible explanations for these observations: (i) *cis*-elements that are important for the missing expression patterns were interrupted when the individual subdomains were isolated or (ii) there are synergistic interactions among subdomains that are essential for the expression patterns. The ability of the subdomains to recreate most of the features of expression of domain B when they are fused to domain A suggests that interactions among subdomains may be responsible for several aspects of the expression pattern. It is interesting to note that expression in the vascular tissue of the hypocotyl can be obtained by two combinations of *cis*-elements. Either B4 in the A domain vector or B4+B5 in the TATA vector consistently confer expression in this tissue.

Discussion

Modular organization of the B domain

We have previously shown that the 35S enhancer can be divided into two domains that confer different expression patterns throughout development (Benfey *et al.*, 1989). A

Fig. 3. Histochemical localization of expression in seedlings of representative plants containing subdomain constructs. (**A**) 4×B1 in 7-day seedling; (**B**) 4×B2 in 7-day seedling; (**C**) 4×B3 in 7-day seedling; (**D**) 4×B4 in 18-day seedling; (**E**) 4×B5 in 7-day seedling; (**F**) 4×B1+A in 7-day seedling; (**G**) 4×B2+A in 7-day seedling; (**H**) 4×B3+A in 7-day seedling; (**I**) 4×B4+A in 7-day seedling; (**J**) 4×B5+A in 18-day seedling; (**K**) A domain in 7-day seedling; (**L**) B domain in 7-day seedling; (**M**) B domain in root of 10-day seedling; (**N**) 4×(B4+B5) in 7-day seedling; (**O**) (B4+B5)+A in 7-day seedling. Abbreviations as for Figure 1 and: M, mesophyll tissue; R, root; Rc, root cap; V, vascular tissue.

fairly simple division of labor appeared to be in effect, the A domain conferred expression principally in root tissue while the B domain conferred expression in all other tissues. Here we show that the B domain can be divided into five subdomains. In Figure 4 we have summarized in schematic form the salient features of the expression patterns of the subdomains fused to either the TATA vector or to the A domain vector. Three of these subdomains are able to confer expression during the early stages of development when fused to a minimal promoter. All five subdomains can give expression when fused to domain A, which differs from the expression of domain A alone. Taken together these findings indicate that there are active *cis*-elements within each of the five subdomains. When analyzed throughout development (see also the accompanying paper) distinct expression patterns for each of the subdomains are detected suggesting that the *cis*-elements found in the different subdomains interact with different *trans*-acting factors. We therefore conclude that the B domain has a modular organization consisting of at least five distinguishable subdomains.

Synergistic interactions among cis-elements

The A domain has been shown to be able to interact synergistically with an upstream sequence (−343 to −208) of the 35S enhancer to activate expression in leaf (Fang *et al.*, 1989) as well as being able to confer expression in root tissue (Poulson and Chua, 1988; Benfey *et al.*, 1989). In order to learn more about the synergistic properties of domain A we analyzed expression from each of the B subdomains fused to this domain. For four of the five subdomains we detected an expression pattern that differed from the pattern of the isolated subdomain or of domain A alone (Figure 4).

In seedlings the expression patterns of the isolated subdomains do not appear to add up to the expression conferred by the intact B domain. One possible explanation is that upon dissection of the B domain, *cis*-elements were interrupted that can confer expression in the tissues that lack expression from the subdomains. An alternative explanation is that synergistic interactions occur among the *cis*-elements found in the B subdomains so that expression is more than the sum of the expression patterns of the isolated elements. Support for the latter possibility comes from the ability of the subdomains to interact synergistically with domain A. The expression patterns that result from these synergistic interactions do add up to the expression pattern of the intact enhancer. In addition, the combination of two subdomains, B4 and B5, gives expression in seeds and seedlings that is not detected with either isolated subdomain. Since this combination also involves uninterrupted sequence it is again possible that a *cis*-element crucial for expression at these two stages was cut when the subdomains were isolated. The fact that the expression pattern of this combination resembles that of B4+A in young seedlings (expression in vascular tissue of the hypocotyl and cotyledons) presents the intriguing possibility that B5 can play a synergistic role similar to that of domain A in combination with subdomain B4. In the accompanying paper we discuss models that can explain the effect on expression of the synergistic interactions among *cis*-elements.

Variation among independent transformants

The use of histochemical localization to detect cell-specific expression patterns is not without potential problems.

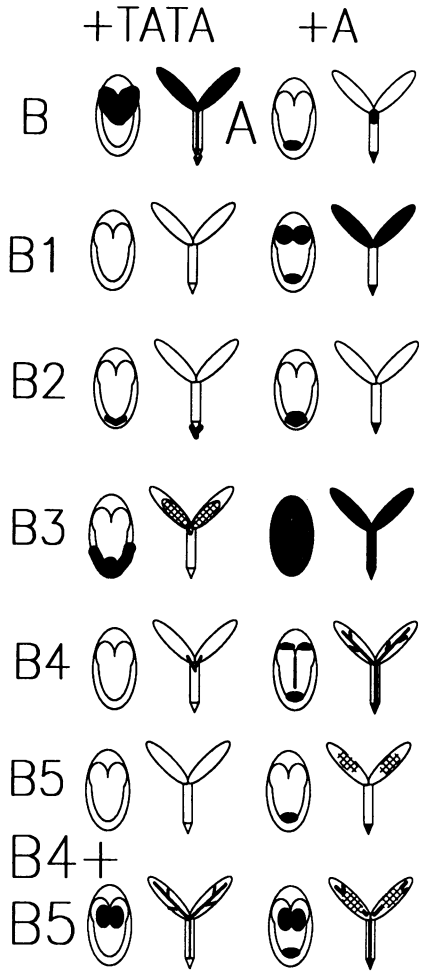

Fig. 4. Schematic representation of expression patterns of subdomain constructs. Expression in seed and seedlings is represented by the darkened areas in the schematic representations of these developmental stages. On the left, expression conferred by the intact B domain (at top) and by the subdomains in the TATA vector is represented. On the right, expression conferred by the domain A vector alone (at top) and by the subdomains in the domain A vector is represented. Only the major features of the expression pattern for each construct have been shown. (For B3+TATA in seeds only one of the two expression patterns is represented.) Cross-hatching represents low level expression in that region.

Differences in cell size and metabolism as well as penetration of the substrate into the cell can contribute to differences in staining intensity (Jefferson *et al.*, 1987). The variety of staining patterns that we have observed is an indication that distinctly different patterns can be detected with these methods. It should be obvious that there may be expression in certain cells from some of the constructs that is below the threshold of detection of the histochemical assay. The intention of this analysis was to define the expression patterns conferred by the subdomains alone or in combination with other *cis*-elements. For this purpose, histochemical localization is as accurate as any method currently available. In Table I we list the number of plants analyzed and the number that gave the expression pattern reported. Except where noted, when a plant did not show the reported expression pattern, no expression in any tissue was observed.

Increasing the copy number of a *cis*-element has been shown in a number of studies to augment expression controlled by the *cis*-element (Ondek *et al.*, 1987; Schirm

Table I. Variation among transgenic plants conaining 35S subdomain constructs

Construct	Stage	Expressing/ analyzed	Variation
4×B1	Sd	0/6	
	Sg	0/6	
4×B1+A	Sd	10/10	2 radicle only
	Sg	11/11	
1×B2+	Sd	0/9	
(−72 to +8)	Sg	0/9	
4×B2(F)	Sd	3/6	
	Sg	4/7	
4×B2(R)	Sd	5/7	
	Sg	5/7	
1×B2+A	Sd	5/8	1 some expression in cotyledons
4×B2(F)+A	Sd	7/10	
	Sg	6/8	
4×B2(R)+A	Sd	9/10	
	Sg	8/9	
4×B3	Sd	7/10	4 strong in radicle pole of endosperm, 3 in cotyledons (see text)
	Sg	7/10	
1×B3+A	Sd	5/11	
	Sg	5/10	
4×B3+A	Sd	10/12	2 cotyledon only
	Sg	9/11	
4×B4	Sd	0/6	
	Sg	1/6	1 plant at 17 days
1×B4+A	Sd	11/13	1 radicle only
	Sg	9/9	
4×B4+A	Sd	10/10	3 radicle only, 1 cotyledon only
	Sg	10/10	
4×B5	Sd	0/8	
	Sg	0/8	
1×B5+A	Sd	10/10	
	Sg	10/10	
4×B5+A	Sd	8/10	
	Sg	8/10	5 mesophyll expression at 18 days
1×(B4+B5)	Sd	4/7	2 some very light expression in radicle
	Sg	1/7	1 plant at 17 days
4×(B4+B5)	Sd	5/11	
	Sg	8/10	
1×(B4+B5)+A	Sd	8/12	
	Sg	11/11	
4×(B4+B5)+A	Sd	3/8	
	Sg	2/6	
B domain	Sd	7/8	
	Sg	7/8	
−46 vector	Sd	0/11	
	Sg	0/11	

Abbreviations: Sd, seed; Sg, seedling; (F), forward orientation; (R), reverse orientation.

et al., 1987; Fromental *et al.*, 1988). We analyzed the effect of multimerization on expression, in the combination of the subdomains with the A domain. In the case of B3, B4 and B5, we found no qualitative difference between one copy and four copies of the subdomain. There was some indication, particularly in the case of B3 and B5 that expression is stronger with the tetramer, but we have not quantified the expression levels. For B2, when the monomer was fused to domain A we did not detect the same pattern of expression as with the tetramer. In order to further assess the role of multimerization of this subdomain we analyzed expression from a construct that contained the monomer fused to the minimal promoter (−72 to +8) used in our previous study (Benfey *et al.*, 1989). No expression was detected at any stage of development. Taken together these results suggest that multimerization of this subdomain is required to detect expression. This raises the possibility that the expression we observed may be due to the creation of new sequences during the multimerization process. We observed that expression in the root cap can be obtained from subdomain B2 as well as from domain B (see also the accompanying paper). This suggests that at least one of the *cis*-elements within this subdomain is also active in the intact domain B. Because of the cell-specificity of expression of this subdomain we analyzed the orientation dependence of expression. We found that subdomain B2 has at least one of the characteristics of an enhancer; it is functional in either orientation.

The two qualitatively different expression patterns that we observed with the B3 subdomain in seeds may indicate the presence of several, possibly overlapping *cis*-elements within this subdomain. It is possible that at different chromosomal insertion sites the *cis*-elements are differentially affected. It is interesting that the intact 35S promoter also shows qualitative differences in expression among independent transgenics (Benfey and Chua, 1989). All of the other subdomain constructs appear to show only quantitative variation among the independent transgenic plants.

Cis-elements and trans-factors

In our effort to understand the modular structure of the 35S enhancer we have defined two domains and five subdomains. Within domain A we have identified a sequence element termed as-1 that binds a factor, ASF-1 (Lam *et al.*, 1989). A cDNA clone that encodes a protein able to bind with the same sequence specificity as ASF-1 has been isolated (Katagiri *et al.*, 1989). The factor is found in nuclear extracts of root and leaf, and the RNA for the cloned factor is higher in root than in leaf (Lam *et al.*, 1989; Katagiri *et al.*, 1989). At this time we cannot conclusively attribute the synergistic properties of domain A to the presence of the as-1 *cis*-element. We note that within subdomain A1 there are also two putative CAAT box sequences. However, we have previously shown that mutation of these sequences within the −343 to +2 promoter does not have any strong effect on expression in any organ (Lam *et al.*, 1989). We also did not detect binding of any factors from nuclear extracts to the putative CAAT box sequences (Lam *et al.*, 1989). In addition, when the as-1 sequence was placed between the TATA region and upstream sequences of a ribulose bisphosphate carboxylase small subunit (rbcS) promoter that normally expresses only in photosynthetic tissue, strong expression in root was observed as well as enhanced expression in leaf tissue (Lam *et al.*, 1989). Further characterization using site-specific mutations, and determination of the distance and orientation dependence of the sequence responsible for the synergistic interaction should allow us to identify the precise sequences involved. The evidence that domain A is able to enhance expression in many tissue types suggests that the factor or factors that mediate this effect are found in most tissues. The fact that

domain A alone is able to confer strong expression only in root tissue suggests that factor interaction is required to mediate expression in other tissues.

Conclusion

In our analysis of expression conferred by subdomains of the 35S enhancer in the early stages of plant development we have shown that the enhancer has a modular organization and that there are synergistic interactions among different *cis*-elements found within the enhancer. Among other enhancers that have been shown to have a modular organization, the simian virus 40 (SV40) promoter is perhaps the best characterized (Ondek *et al.*, 1987; Schirm *et al.*, 1987; Fromental *et al.*, 1988). In this case numerous *cis*-elements have been characterized; however, the contribution of each of these elements to expression throughout development and in more than a few cell types has not been analyzed. In the accompanying paper (Benfey *et al.*, 1990) we present the results of analysis of expression from the 35S enhancer subdomains in mature plants. The tissue-specific expression patterns that are observed provide insight into the interactions among *cis*-elements that define expression throughout development.

Materials and methods

Constructs

The construct containing domain A into which the subdomains were inserted is the same as X-GUS-90 described previously (Benfey and Chua, 1989). This is referred to as the 'A domain vector'. The minimal promoter construct (X-GUS-46) was made in essentially the same manner except that the fragment from −46 to +8 was synthesized as complementary oligonucleotides with a *Hin*dIII site at the 3′ end and a *Bgl*II site at the 5′ end. This was subcloned, sequenced for accuracy and then fused to the GUS coding region. The *Hin*dIII site was filled in with Klenow enzyme. The *Bgl*II (5′)−*Eco*RI (3′) fragment containing the 35S −46 to +8 sequence fused to the GUS coding sequence with the 3′ polyadenylation sequence from pea *rbcS3C* was then inserted between the *Bgl*II and *Eco*RI sites of the polylinker of pMON505 (Horsch and Klee, 1986). This vector is designated as the 'TATA vector'. A construct containing a 35S upstream fragment (−941 to +8) fused to the chloramphenicol acetyl transferase (CAT) coding sequence with the 3′ polyadenylation sequence from pea *rbcSE9* was inserted as a blunt end ligation into the *Hpa*I site 4 kb away from the GUS construct. (CAT activity was routinely measured to confirm that plants were transformed.) Four subdomains (B1, B2, B3 and B5) were synthesized as complementary oligonucleotides. The B4 subdomain was cut from a 3′ deletion derivative of the 35S promoter described in Fang *et al.* (1989) from −343 to −208 by cleavage with *Hin*fI at nucleotide −301. Tetramers were made by head to tail ligations of monomers, except for B1 which was initially synthesized only as a tetramer. Monomers and tetramers were inserted between the *Hin*dIII (5′) and *Xho*I (3′) sites in X-GUS-90 and in X-GUS-46, except for the tetramer of B2 which was inserted in either orientation in the *Xho*I site and the tetramer of B4 which was inserted between the *Hin*dIII (5′) and *Sac*I (3′) sites of both vectors. The combination of B4 and B5 was a fragment from −343 to −208 described in the deletion analysis of the 35S promoter in Fang *et al.* (1989) with an *Xho*I site at the 5′ end and a *Sal*I site at the 3′ end. This was multimerized by ligation of the fragment at high concentration. It was subcloned into a derivative of pEMBL13 at the *Sal*I site and inserted into the expression vectors between the *Hin*dIII site (5′) and *Xho*I site (3′). Further details of the cloning are available upon request.

Transgenic plants

The constructs were mobilized into *Agrobacterium tumefaciens* and transgenic tobacco plants were generated as previously described (Benfey *et al.*, 1989). Seeds and seedlings were handled as previously described (Benfey *et al.*, 1989).

Histochemical staining

Histochemical staining was performed as previously described (Jefferson *et al.*, 1987; Benfey *et al.*, 1989).

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