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ASF-2: A Factor That Binds to the Cauliflower Mosaic Virus 35S Promoter and a Conserved GATA Motif in *Cab* Promoters

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We have used nuclear extracts prepared from tobacco leaf tissue to characterize a factor binding site, designated *as-2* (activating sequence-2), at the −100 region of the cauliflower mosaic virus 35S promoter. The activity of this factor, called ASF-2 (activating sequence factor-2), is not detected in tobacco root extracts. *as-2* includes two GT motifs with sequence homology to the SV40 enhancer core A element and the Box II element of pea *rbcS*. Nevertheless, oligomers of these sequence elements do not compete for ASF-2 binding in gel retardation assays, indicating that the GT motifs may not be involved. Methylation interference studies identify two guanines (G93 and G98) that are required for interaction with ASF-2. Sequences surrounding these two critical guanines display homologies to a GATA repeat conserved among several light-responsive promoters. One such sequence from a petunia *Cab* promoter is able to compete with *as-2* for factor binding. In transgenic plants, a tetramer of *as-2* is able to confer leaf expression when fused 5′ to the −90 derivative of the 35S promoter. The expression is not dependent on light and, thus, the *as-2* tetramer does not function as a light-responsive element in this context. Histochemical localization of the reporter gene product suggests that the *as-2* tetramer directs expression in trichomes, vascular elements, and epidermal and mesophyll cells.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is a double-stranded DNA virus that infects many members of the Cruciferae. In the infected plant cells, the virus produces two major transcripts of 19S and 35S in size (Guilley et al., 1982). The 35S promoter has been shown to function as a strong promoter in various organs of transgenic plants (Odell, Nagy, and Chua, 1985; Kay et al., 1987; Benfey and Chua, 1989; Fang et al., 1989), as well as in protoplasts of monocots and dicots (Fromm, Taylor, and Walbot, 1985; On-Lee, Turgeon, and Wu, 1986; Ow, Jacobs, and Howell, 1987; Odell et al., 1988). Because expression of the 35S promoter does not require any viral-encoded protein (Odell et al., 1985), host nuclear factors must be responsible for its activity.

The high expression level and apparent constitutive nature of the 35S promoter have prompted us to initiate a project aimed at detailed characterization of its regulatory elements and their cognate factors. We found that the promoter can be divided into at least two domains, A and B, each with a distinct cell specificity (Benfey, Ren, and Chua, 1989). Domain A (−90 to +8) confers preferential expression in root, and domain B (−343 to −90) in leaf. To identify *cis*-acting elements responsible for cell specificity, we have localized nuclear factor binding sites on the 35S promoter by DNase I footprinting (Green et al., 1989). Using this approach, we have identified activating sequence (*as*)-1, which encompasses the sequence between −85 and −59 and which interacts with a factor designated ASF-1 (Lam, Benfey, and Chua, 1989a; Lam et al., 1989b). From site-specific mutation analyses, ASF-1 appears to be important for expression of the 35S promoter in root cells, as well as in protoplasts prepared from tobacco leaves (Lam et al., 1989a, 1989b).

In this paper we describe the characterization of a second factor, ASF-2, which binds to the −100 region of the 35S promoter, designated *as-2*. Binding activity of ASF-2 can be detected in leaf but not in root nuclear extracts. By methylation interference assays we have identified two guanines, at positions −93 and −98, critical for DNA-protein interaction. Mutations of these two critical guanines lead to impaired ASF-2 binding. In transgenic plants *as-2* tetramer does not appear to confer any significant expression in root. However, histochemical localization of the reporter gene product, β-glucuronidase (GUS), reveals that the tetramer confers expression in all leaf cell types. ASF-2 also binds to a conserved GATA repeat found

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in the promoters of *Cab* and other light-responsive genes. Our results suggest that the 35S promoter and the *Cab* promoters are regulated by at least one common factor.

RESULTS

Characterization of a Factor, ASF-2, That Binds to the CaMV 35S Promoter

We have shown previously that nuclear extracts from pea and tobacco contain a protein factor ASF-1. This factor binds to a sequence, designated *as-1*, at the −75 region of the CaMV 35S promoter (Lam et al., 1989a, 1989b). Figure 1A shows a comparison of the DNaseI footprint on the lower strand of the 35S promoter obtained with pea whole cell extract and tobacco nuclear extract. At lower extract concentrations, the tobacco extract preferentially protects a region of the 35S promoter upstream from *as-1* (Figure 1A, lane 2). At higher extract concentrations *as-1* is also protected (Figure 1A, lane 1). In contrast, whole cell extracts from pea (Figure 1A, lane 3) or nuclear extracts from pea (data not shown) do not show any obvious protection in this region other than *as-1*. The protected region observed with low concentrations of tobacco nuclear extract is designated *as-2* for activating sequence-2 (Figure 1B). Protected residues for the upper strand of the 35S promoter were mapped by similar methods (data not shown) as in Figure 1A.

In previous studies (Lam et al., 1989a, 1989b) we have shown that binding of ASF-1 requires two TGACG motifs in the −75 region of the 35S promoter. A TGACG motif is also found in a conserved hexameric motif in the promoters of histone genes, and a wheat nuclear factor, HBP-1, has been reported to bind to this hexameric motif in vitro (Mikami et al., 1987). To demonstrate that the regions of the 35S promoter protected by increasing amounts of tobacco nuclear extract represent binding of two distinct factors, we have carried out competition experiments with the wheat histone H3 promoter fragment. Figure 1C shows that ASF-1 in pea extracts can be competed by the histone H3 promoter fragment containing the HBP-1 binding site. With tobacco nuclear extracts, protection of the *as-1* region but not of the *as-2* region is competed by the same fragment. Moreover, the 35S promoter mutant *as-1c*, which has a 2-bp mutation at each of the TGACG motifs (Lam et al., 1989a, 1989b), does not compete at the same molar excess (data not shown). We have shown previously that this mutant is impaired in ASF-1 binding in vitro (Lam et al., 1989a, 1989b). Taken together, these results provide evidence that ASF-2 is distinct from ASF-1.

Examination of *as-2* shows that it contains sequences homologous to both the core A enhancer element of SV40 (Ondek, Shepard, and Herr, 1987) and the plant GT-1 binding site, Box II, of *rbcS* (Green, Kay, and Chua, 1987).

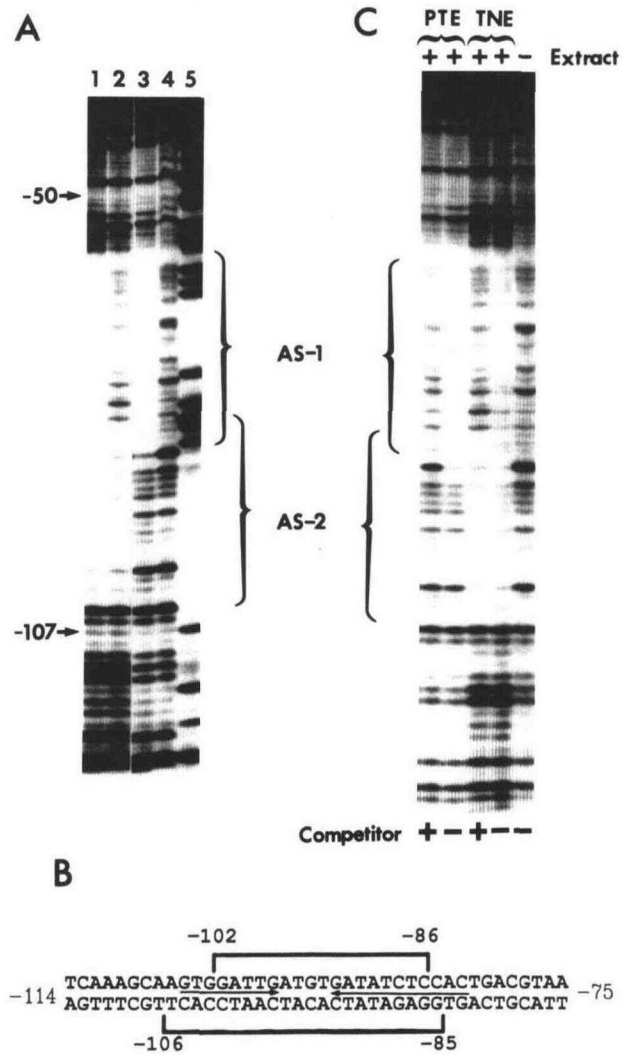

Figure 1. Characterization of Factors Binding to the Upstream Region of the 35S Promoter.

(A) DNase I footprints of different extracts on the 35S promoter. A probe was prepared by Klenow fill-in with ^{32}P nucleotides at the lower strand of the −130 to +8 fragment of the 35S promoter. Subsequently, the insert was isolated by restriction enzyme digestion and gel electrophoresis. Lane 1, 20 μL of tobacco nuclear extract; lane 2, 10 μL of tobacco nuclear extract; lane 3, 8 μL of pea whole cell extract; lane 4, no extract; lane 5, position of guanines determined by the method of Maxam and Gilbert (1980). The protein concentration was 20 μg/μL for the tobacco nuclear extract and 40 μg/μL for the pea whole cell extract.

(B) The nucleotide sequence of the 35S promoter from −114 to −75. Brackets indicate the regions protected from DNase I digestion when incubated with 10 μL of tobacco nuclear extract. The putative GT motifs are underlined with arrows.

(C) Competition analysis with a fragment from the wheat histone H3 promoter. Eight μL of pea whole cell extract (PTE) and 20 μL of tobacco nuclear extract (TNE) were used where indicated. The −183 to −100 fragment of the wheat histone H3 promoter (Mikami et al., 1987) was added as a competitor at 250 molar excess.

These sequences, underlined in Figure 1B, are located at the borders of the ASF-2 protected region. To determine whether ASF-2 also binds to the SV40 core A element or to Box II, we carried out competition experiments using tetramers of these elements. Figure 2 shows a gel retardation assay with tobacco nuclear extract and the 35S promoter as probe. At the low extract concentration used in this assay, only ASF-2 but not ASF-1 binding is observed because the mutant 35S promoter *as-1c* shows the same gel shift band (data not shown). Under these conditions, the DNA-protein complex observed can be efficiently competed by 100-fold molar excess of non-labeled 35S promoter fragment. In contrast, the multimerized SV40 core A element or the Box II tetramer does not compete significantly, even at higher molar excess. From these results we conclude that ASF-2 does not bind to the SV40 core A element or to Box II.

To identify the guanines critical for the interaction of ASF-2 with its binding site, we carried out methylation interference assays under the same conditions as for gel retardation experiments. Figure 3 shows that methylation of either of the two guanines, G98 and G93 on the upper strand of the 35S promoter, severely blocks ASF-2 binding

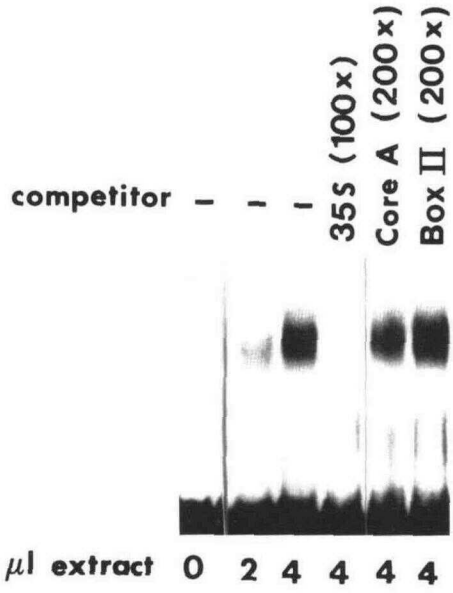

Figure 2. Competition Analysis of ASF-2 with GT Motif Binding Sites.

The amount of tobacco nuclear extract (15 $\mu g/\mu L$) added is shown at the bottom. The binding probe was the same as that used for DNase I footprinting experiments in Figure 1. The −130 to +8 fragment of the 35S promoter and tetramers (84 bp) of core A and Box II elements were used as competitors. Free DNA and the DNA-protein complex were resolved with an agarose/polyacrylamide composite gel (Mikami et al., 1987).

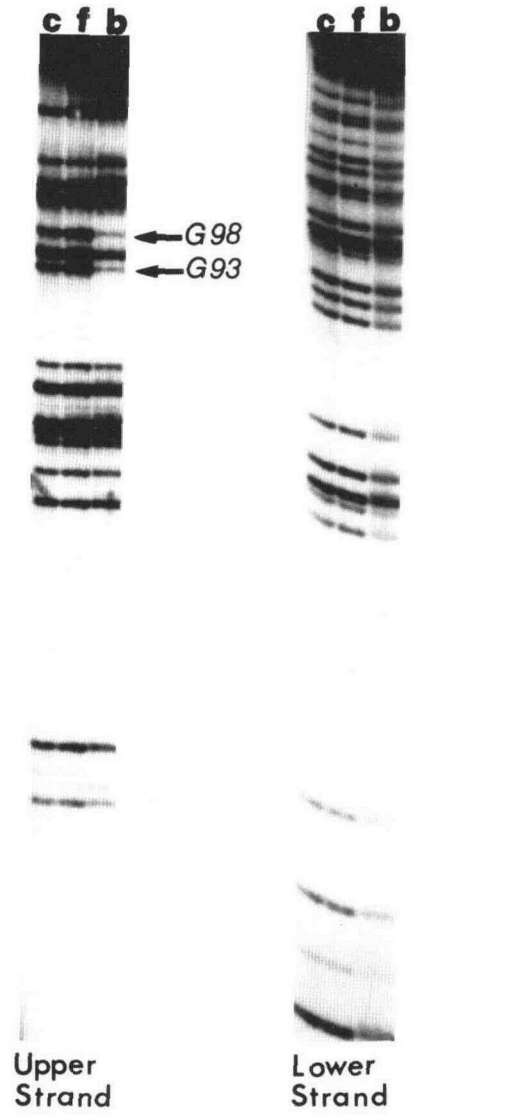

Figure 3. Methylation Interference Assay for Critical Guanines in *as-2*.

Preparative gel retardation assays were carried out with partially methylated probes radiolabeled at either the upper or lower strand of the −130 to +8 fragment of the 35S promoter. After electrophoresis, the free and bound complexes were electroeluted and then cleaved with piperidine. The cleaved products were separated by electrophoresis on a 6% urea/polyacrylamide gel. Lane c, control methylated probe before gel retardation assay; lane f, free probe; lane b, DNA fragment complexed with ASF-2. G93 and G98 refer to the guanine residues at −93 and −98 of the upper strand, respectively (see Figure 1B). For positions of the guanines of the lower strand, see lane 5 of Figure 1A. Some of the bands at lane b of the lower strand are rather diffuse, and appear to be reduced in intensity. However, they are not reproducible, and represent artifacts of the particular assay.

in vitro. On the other hand, methylation of any one guanine in the lower strand does not cause an obvious decrease in ASF-2 binding. These results suggest that the guanines at positions −93 and −98 are important for sequence-specific binding of ASF-2. To confirm this interpretation, we synthesized tetramers of the wild-type *as-2* sequence and a mutant derivative, *as-2b*, in which guanines G98 and G93 were replaced by cytosine and adenine, respectively. The multimers were synthesized as 21-bp direct repeats to place the factor on the same face of the DNA helix and, thus, maximize any possible cooperative interaction between adjacent factors. These probes were used in gel retardation assays with tobacco leaf and root nuclear extracts. As shown in Figure 4, the *as-2* tetramer, but not the *as-2b* tetramer, forms a complex similar in mobility to that observed with the 35S promoter fragment. These results confirm the importance of G93 and G98 in ASF-2 binding. A complex with faster mobility is also observed with the tobacco leaf nuclear extract used in this assay. This complex is not affected by unlabeled *as-2* when added as competitor (data not shown), and, thus, likely represents a nonspecific DNA binding activity in some of our extracts. The similarity in mobility of the ASF-2 DNA-protein complex with the tetramer as compared with that observed with a single *as-2* binding site suggests a lack of cooperativity in ASF-2 binding. No complex is detected with root nuclear extracts using either *as-2* or *as-2b* as a probe. This same root extract contains ASF-1, as was shown previously (Lam et al., 1989b). It should be noted that the root nuclear extract (5 μg total) is more dilute than the leaf nuclear extract (20 μg total). However, diluting the leaf nuclear extract in the gel retardation assay to 5 μg total still allows the detection of ASF-2 binding activity (data not shown). Thus, the apparent lack of ASF-2 binding activity is not the result of lower protein concentration in the root nuclear extract. At present, however, we cannot exclude the possibility that ASF-2 is more labile than ASF-1 during extract preparation from tobacco root.

Examination of the sequence surrounding the critical guanines in *as-2* reveals a tandem repeat of TGAT that is located at the center of the region protected from DNase I digestion (Figure 1B). There is also a similarity between *as-2* and a conserved GATA repeat that has been reported to be conserved among promoters of light-responsive genes, particularly the *Cab* promoters (Castresana et al., 1987; Grob and Stuber, 1987; Gidoni et al., 1989). To investigate whether ASF-2 can bind to the conserved GATA motif from *Cab* promoters, we synthesized a DNA fragment corresponding to the −75 to −59 region of the petunia 22L *Cab* gene (Gidoni et al., 1989), which contains the GATA repeat sequence. The cloned DNA fragment was used in gel retardation and competition assays. Figure 5 shows that the 22L *Cab* GATA sequence can compete specifically with the *as-2* tetramer, whereas the mutant *as-2b* tetramer cannot (left panel). Although the 22L *Cab* fragment appears to compete less efficiently at 50-fold

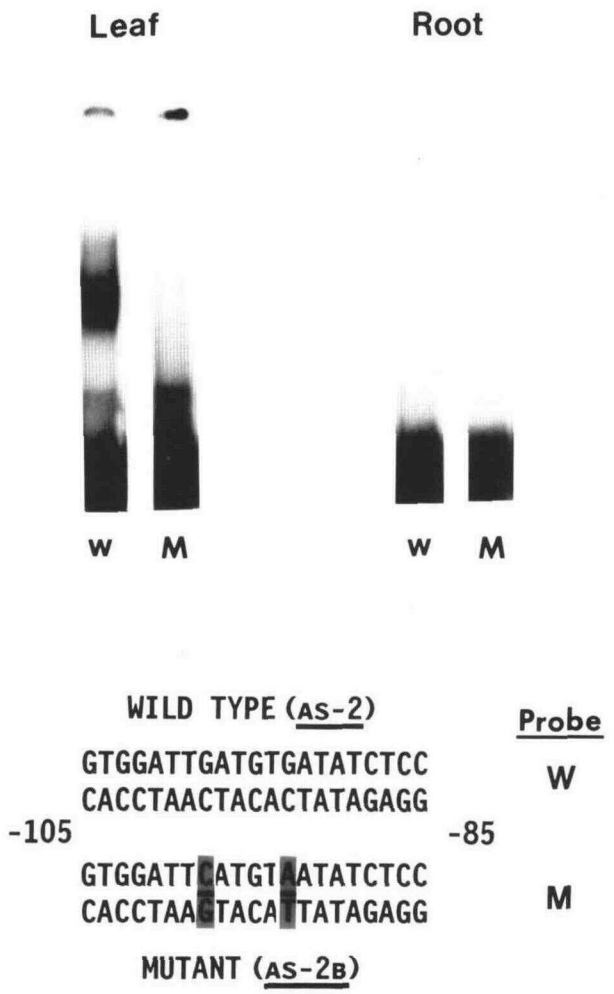

Figure 4. ASF-2 Is Detected in Leaf but not Root Nuclear Extracts.

The sequence from −105 to −85 (*as-2*) of the 35S promoter was synthesized as a tetramer (W). The same tetramer sequence but mutated at guanines 98 and 93 (*as-2b*) was also synthesized (M). *as-2* and *as-2b* were cloned into pEMBL12 derivatives and used as probes for gel retardation assays. Left panel, 4 μL (20 μg of protein) of nuclear extract from tobacco leaf; right panel, 10 μL (5 μg of protein) of nuclear extract from tobacco root.

molar excess as compared with the *as-2* sequence, it should be noted that the *as-2* tetramer contains four times more binding sites for ASF-2 as compared with the 22L *Cab* fragment. Indeed, when 4 times as much 22L *Cab* fragment is used, i.e., at 200-fold excess, a similar level of competition is observed as with 50-fold excess of *as-2* tetramer. These results indicate that the two sequences, in fact, may bind to ASF-2 with similar affinity. When the 22L *Cab* fragment is used as a probe for gel retardation

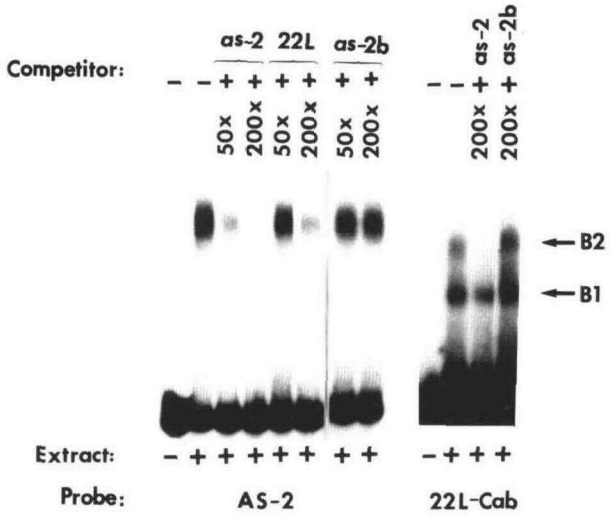

Figure 5. The GATA Sequence Element in the Petunia 22L *Cab* Promoter Binds ASF-2.

Gel retardation analysis was carried out with either the *as-2* tetramer probe (95 bp) (left panel) or a fragment containing the cloned petunia 22L *Cab* GATA sequence (64 bp) as a probe (right panel). The cloned petunia *Cab* sequence, 5′-GTAGATAGATATCAT-3′ [from −75 to −59 of 22L *Cab* (Gidoni et al., 1989) where the GATA repeats are underlined], is inserted into pEMBL12 derivatives and is isolated and labeled as a HindIII/EcoRI fragment. For each lane with extract, 4 μL of tobacco nuclear extract (20 μg of protein) was used. Where indicated, the unlabeled fragments of the *as-2* tetramer (as-2), the *as-2b* tetramer (as-2b), or the 22L *Cab* GATA element (22L) were added as competitors.

assay, at least two distinct complexes can be observed with tobacco leaf extracts (Figure 5, right panel). The slower migrating complex (B2 in Figure 5) can be competed efficiently by the *as-2* tetramer, but not by the *as-2b* tetramer. The faster migrating complex, B1, is not competed by the *as-2* tetramer and, thus, may involve factors distinct from ASF-2. These data suggest that ASF-2 can bind to the conserved GATA motif in the *Cab* promoters, but the GATA sequences from the *Cab* promoters probably interact with factors in addition to ASF-2.

Functional Definition of *as-2*

The presence of a binding site for ASF-2 in the CaMV 35S promoter as well as in *Cab* promoters raises the question of its function in vivo. To address this question, we examined expression conferred by the *as-2* tetramer in transgenic plants. The mutant *as-2b* tetramer was used as a negative control because it does not bind ASF-2 in vitro. We inserted the wild-type and mutant *as-2* sequences upstream of the −46 to +8 (X-GUS-46 vector) or −90 to +8 (X-GUS-90 vector) derivatives of the 35S promoter. The reporter gene GUS was placed downstream from the +8 site of the 35S promoter to assay for expression. Table 1 shows representative GUS activity from root and leaf extracts of independent transgenic tobacco plants (cv SR1) containing the different constructs. The values observed with X-GUS-46 are essentially identical with those obtained with nontransformed tobacco plants and, thus, represent background activities. The *as-2* tetramer does not show any significant activity when fused 5′ to the −46 derivative of the 35S promoter. However, when fused 5′ to the −90 derivative of the same promoter, GUS activity is expressed in leaf of the transgenic plants. Control transgenic plants with either the X-GUS-90 vector alone or with the tetramer of the *as-2b* mutant (4AS2m-90 construct) show little or no GUS activity above background. In roots, the −90 derivative of the 35S promoter shows clear activity on its own, and this activity can be enhanced by about 10-fold when the upstream element (−343 to −90) of the 35S promoter is fused directly 5′ (Benfey et al., 1989). However, there is little increase in root expression of the reporter gene when either the *as-2* or the *as-2b* tetramer is fused to the −90 derivative of the 35S promoter. This observation indicates that four tandem ASF-2 binding sites can act as a positive element to drive expression preferentially in leaf as compared with root. This leaf expression is apparently dependent on sequences located within the −90 to −46 region of the 35S promoter.

To examine the cell type-specific expression of the *as-2* tetramer, we carried out histochemical localization of the

Table 1. Activity of *as-2* Tetramers in Transgenic Tobacco

Construct	Expression	
	Root	Leaf
X-GUS-46	280	50
4AS2-46	325	49
4AS2m-46	372	62
X-GUS-90	1825	70
4AS2-90	1902	1238
4AS2m-90	1483	156

Activity is presented in picomoles of 4-methylumbelliferone per minute per milligram of protein. Five to ten independent transformants were analyzed for each construct, and data from a representative plant are shown. For each construct, the activities of independent transformants showed a variation of no more than threefold around the mean. *as-2* and *as-2b* mutant tetramers were cloned into the X-GUS-46 vector via the unique XhoI and HindIII site to give the constructs 4AS2-46 and 4AS2m-46, respectively. For the 4AS2-90 and 4AS2m-90 constructs, the X-GUS-90 vector was used instead of the X-GUS-46 vector. Activities observed with plants transformed with the X-GUS-46 vector are similar to those obtained with nontransformed *Nicotiana tabacum* cv SR1 and, thus, represent background GUS activities.

GUS gene product in leaf and root sections of transgenic tobacco containing the 4AS2-90 construct (Jefferson, Kavanagh, and Bevan, 1987). Figure 6 shows that all distinguishable leaf cell types appear to express the reporter gene product. The most intensely stained cell types are localized to the vascular tissues of the leaf. In addition, the epidermis as well as the trichomes are also readily stained. We consistently found that the spongy mesophyll cells are more highly stained than the palisade mesophyll cells. Examination of plants containing the *as-2b* tetramer (4AS2m-90) shows no detectable staining in all the leaf cell types (data not shown). From these results we conclude that the observed staining pattern likely depends on ASF-2 or a factor with similar binding specificity.

Figure 6F shows the staining pattern of a root from a transgenic plant with the 4AS2-90 construct. Clear and intense staining can be observed at the root tip region around the meristem, with little staining elsewhere in the root. This is the same expression pattern reported previously for the −90 derivative of the 35S promoter (Benfey et al., 1989). Because this expression pattern is also observed in the roots of transgenic plants containing the *as-2b* tetramer construct (data not shown), it is likely to be conferred by the −90 35S promoter alone and not by the binding of factors to the *as-2* sequence. Our data suggest that *as-2* tetramer can confer preferential leaf cell expression in conjunction with the −90 element of the 35S promoter.

Because the GATA sequence is found in light-responsive promoters, we investigated whether expression of the 4AS2-90 construct is light-responsive. Total RNA prepared from light-grown and dark-adapted transgenic plants were analyzed by S1 nuclease protection assays (Figure 7). We found no significant light induction of the GUS transcript levels in independent transformants. In several cases (i.e., transgenic plant 2 and 3 in Figure 7), the level of GUS mRNA is even higher in the dark when compared with the level in the light. Our results thus suggest that *as-2* tetramer is not a light-responsive element.

DISCUSSION

Characterization of ASF-2

In this paper we describe the characterization of a second tobacco nuclear factor that binds to the CaMV 35S promoter. This factor, called ASF-2, binds to the −100 region of the 35S promoter and is distinct from ASF-1, which binds to sequences between −85 and −59 of the same promoter (Lam et al., 1989a, 1989b). *as-2*, the binding site for ASF-2, encompasses sequences homologous to the SV40 core A enhancer (Odell et al., 1985; Ow et al., 1987) and *rbcS* Box II (Green et al., 1987). However, competition experiments and methylation interference assays show that ASF-2 does not interact with these elements. Our results demonstrate that ASF-2 probably binds to a GATA repeat that is conserved among *Cab* promoters from diverse plant species.

Figure 8 compares sequences surrounding the GATA motif of several *Cab* promoters with that of *as-2* from the 35S promoter. The sequences are aligned at the proximal GATA motif that is conserved in all the sequences. The distal GATA motif is more variable, and some of the sequences, including that of the 35S *as-2* site, have one nucleotide mismatch with the GATA motif. An interesting feature that emerged from this comparison is that, whereas all *Cab* promoters shown have two nucleotides between the two GATA motifs, the *as-2* site has only one nucleotide. This difference may be responsible for the additional DNA-protein complex seen with the *Cab* GATA sequence in gel retardation assays (B1 in Figure 5). Other differences in the sequences adjacent to the GATA boxes may also contribute to the additional complex, B1, found with the *Cab* sequence. Computer searches of the data bank carried out by Grob and Stuber (1987) have uncovered a sequence element with a GATA motif, which is found in most of the light-responsive promoters. This observation suggests that ASF-2 or related GATA-binding factors may be involved in the expression of light-responsive genes in general. However, ASF-2 per se does not appear to be sufficient to confer light-responsiveness because the expression of 4AS2-90 is not light-dependent. We note that the light-responsive genes searched in the work of Grob and Stuber (1987) are mostly expressed in leaf. Therefore, we consider it likely that ASF-2 may mediate leaf-specific expression of these promoters rather than be involved directly in their light-responsiveness. In this context, we note that the promoters for *rbcS* and *Cab* are expressed preferentially in chloroplasts-containing cells (Aoyagi, Kuhlemeier, and Chua, 1988). Because *as-2* apparently lacks cell specificity, at least one additional factor other than ASF-2 must be responsible for the cell-specific expression of *rbcS* and *Cab*.

Functional Definition of *as-2*: Architecture of the CaMV 35S Promoter

To define the function of ASF-2, we demonstrate that a tetramer of its binding site, *as-2*, can confer preferential leaf expression when fused 5′ to the −90 derivative of the 35S promoter. This activity is correlated with ASF-2 binding, as a 2-bp mutation in *as-2* reduces binding of ASF-2 in vitro and severely diminishes its activity in vivo. We must emphasize that the activity attributed to *as-2* requires multimerization of the element because the −105 derivative of the 35S promoter, which contains only one copy of *as-2*, is not active in leaf (Poulsen and Chua, 1988). This

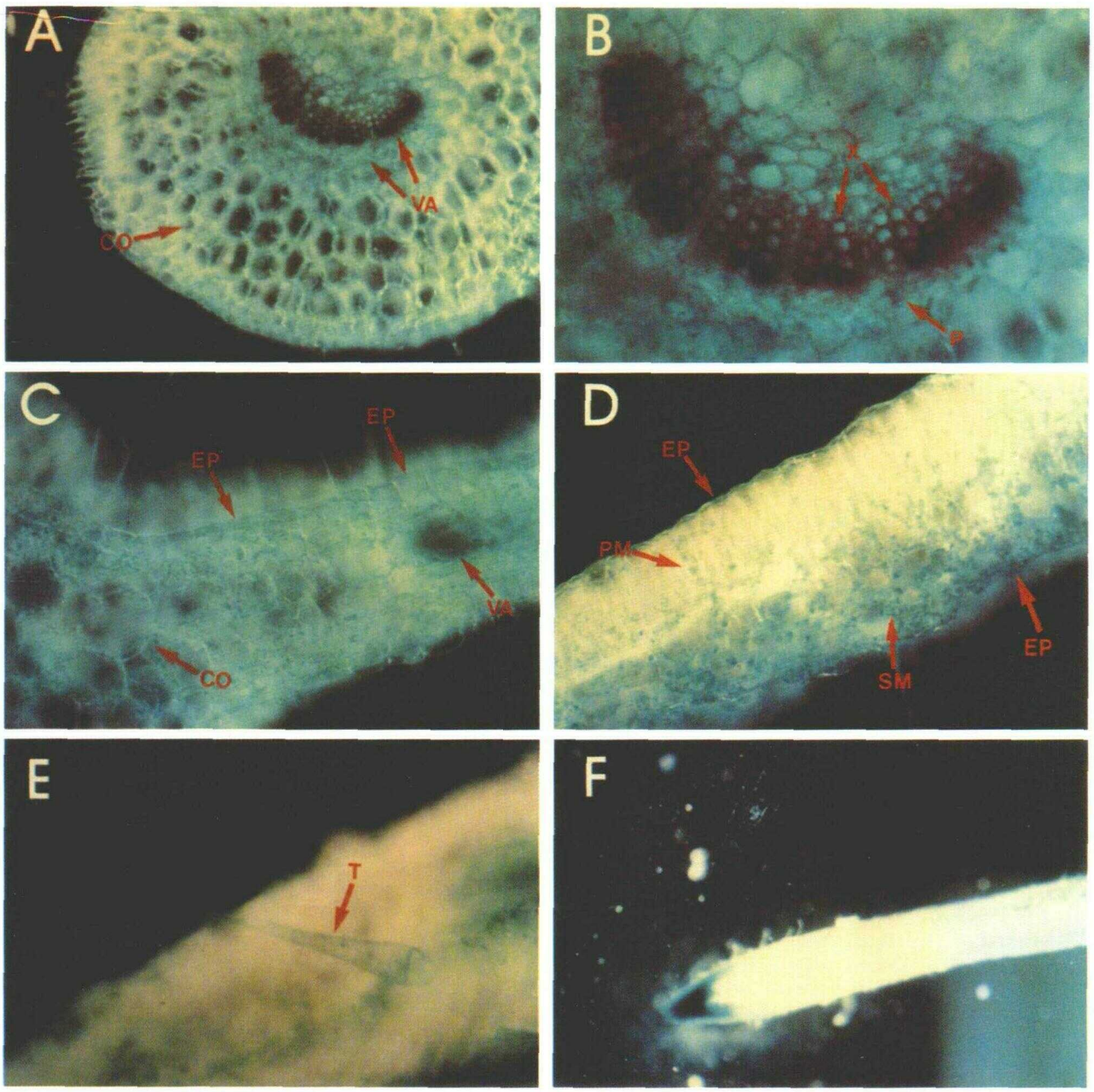

Figure 6. *Histochemical Localization of GUS Expression in Transgenic Tobacco.*

Transgenic tobacco plants expressing the 4AS2-90 construct were used for histochemical assay for GUS expression.
(A) Cross-section of the midvein of a mature leaf.
(B) Close-up of the central vascular element of the midvein.
(C) Cross-section of the lamina regions of the leaf close to the midrib.
(D) Cross-section of the lamina region of the leaf further out toward the edge of the leaf.
(E) Highlight of a stained trichome against an incompletely stained leaf section in the background.
(F) A longitudinal section of a root.
EP, epidermis; VA, vascular elements; CO, collenchyma; PM, palisade mesophyll cells; SM, spongy mesophyll cells; T, trichome; P, phloem; X, xylem.

Figure 7. *as-2* Tetramer Does not Confer Light-Responsiveness.

Leaf samples from transgenic tobacco plants with the 4AS2-90 construct are collected from light-grown, mature plants (L) and the same plants after dark-adaptation for 2 days (D). Total RNA was prepared, and the expression of GUS transcripts was quantitated by S1 nuclease protection. Ten μg of RNA was used for each lane, and data from four independent transformants are shown. Only the protected signals for the test gene are shown. RNA from leaves of tobacco transformed with the X-GUS-90 vector or with the 4AS2m-90 construct did not show any significant amount of the protected signal for the test gene (data not shown).

observation is reminiscent of previous studies with the SV40 promoter elements in mammalian cells, where multimerization greatly enhances activity of individual elements (Ondek et al., 1987; Schirm, Jiricny, and Schaffner, 1987; Fromental et al., 1988). The activity of *as-2* tetramer depends on sequences between −90 and −46 of the 35S promoter. This likely reflects a dependence for ASF-1 binding to the −75 region, although site-specific mutation of the ASF-1 binding site is necessary to confirm this hypothesis. Taken together, these results suggest that *as-2* belongs to the class B type of enhancer motif defined by Fromental et al. (1988), which can activate transcription only in association with a second motif.

The observation that root extracts from tobacco appear to have little detectable ASF-2 is consistent with the observation that *as-2* tetramer has no significant activity in roots of transgenic plants. Thus, ASF-2 activity appears to be primarily localized in leaf relative to root. Since the *as-2* tetramer does not confer any dramatic cell-type specificity in leaf, it may be an organ-specific rather than a cell type-specific element. It is intriguing that we have failed so far to detect any ASF-2 activity in pea whole cell or nuclear extracts. One possibility is that ASF-2 may be more labile in pea and is readily inactivated during extract preparation. Alternatively, ASF-2 may not be ubiquitous in the plant kingdom and is absent from pea. In this regard, we note that the pea AB80 *Cab* promoter does not contain the conserved GATA repeat in the same location as in most of the other *Cab* promoters (Castresana et al., 1987; Gidoni et al., 1989).

Previous studies by our laboratory have shown that the 35S promoter contains at least two domains. Domain B, which is upstream of −90, can confer expression in leaf and stem, whereas domain A, which is downstream from −90, can confer expression preferentially in root (Poulsen and Chua, 1988; Benfey et al., 1989; Fang et al., 1989). Our work on ASF-1, a factor that binds to the −75 region of the 35S promoter, suggests that a tandemly repeated TGACG motif is required for high levels of root expression (Lam et al., 1989b). In this paper we have characterized a second factor, ASF-2, which binds immediately upstream of *as-1* in the 35S promoter. *as-2* is part of domain B, which is responsible for leaf expression of the 35S promoter (Benfey et al., 1989), and, interestingly, a tetramer of this binding site also confers expression preferentially in leaf. However, our deletion studies show that *as-2* can be deleted without a severe loss in leaf expression [i.e., less than 50% (Fang et al., 1989)]. Therefore, other elements present upstream of *as-2* in the 35S promoter must also be involved in leaf expression. Future definition of these other elements will enhance our understanding of the architecture of this strong promoter and uncover the rules governing the interaction among the various factors to generate cell-specific gene expression.

METHODS

Preparation of Plant Extracts and Analysis of DNA-Protein Interactions

Crude nuclear extract from tobacco was prepared essentially according to Green et al. (1987) except that the Percoll gradient

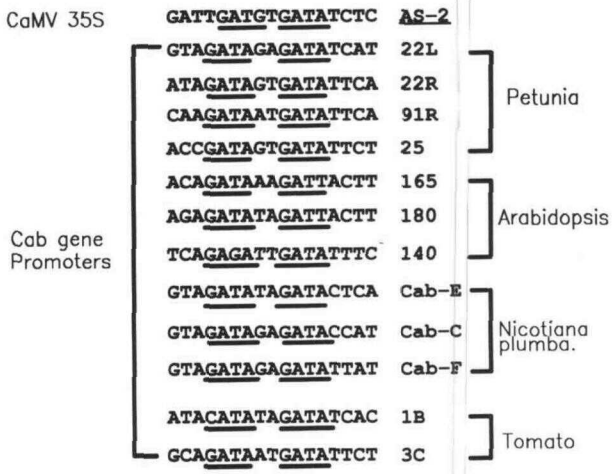

Figure 8. Comparison of Related Sequences in *Cab* Promoters and *as-2*.

The *Cab* promoter sequences are from Castresana et al. (1987) and Gidoni et al. (1989). The GATA repeats are underlined.

step was omitted. Whole cell extract was prepared from peas as described in Lam et al. (1989b). DNase I footprinting was carried out under the conditions described in Green et al. (1987) with the modifications described in Lam et al. (1989b). Methylation interference assay was carried out as described in Green et al. (1989). Gel retardation assay was performed as described by Mikami et al. (1987).

Cloning Vectors, Binding Sites, and Construction of Transgenic Plants

The X-GUS-90 vector has been described in Benfey and Chua (1989). The X-GUS-46 vector is similar to X-GUS-90 except that the 5′ end point of the 35S promoter is at −46 instead of −90. Both vectors contain unique XhoI and HindIII sites at the 5′ border of the truncated 35S promoters. For the polyadenylation signal, a 3′ nontranslated sequence from pea *rbcS-3C* (Fluhr et al., 1986) was fused to the 3′ end of the GUS coding sequence. Tetramers of the *as-2* and *as-2b* sequences were synthesized as oligonucleotide pairs and were cloned and sequenced in pEMBL12 derivatives at the XhoI and HindIII sites. These inserts were then transferred into the X-GUS-90 and X-GUS-46 vectors. Transgenic tobacco plants were obtained by transformation of tobacco (cv SR1) leaf discs with *Agrobacterium tumefaciens* harboring these pMON505 derivatives (Fraley et al., 1985). The oligonucleotide pairs for the petunia 22L *Cab* GATA box sequence (5′-AGCTTGTCGACGTAGATAGAGATATCATAGATCTC-3′, top strand; 5′-TCGAGAGATCTATGATATCTCTATCTACGTCGACA-3′, lower strand) were synthesized, annealed, and then cloned into pEMBL12 derivatives for sequencing and plasmid preparation.

Analysis of Transgenic Plants

GUS activity was assayed with soluble extracts from root and leaf of mature transgenic tobacco plants essentially as described (Jefferson et al., 1987; Benfey et al., 1989). Two time points were obtained for each assay to ensure linearity of the measured enzymatic activity. Histochemistry of hand-sectioned organs of transgenic tobacco plants was carried out as described by Benfey et al. (1989). Total RNA was isolated and analyzed by S1 nuclease protection assays as described previously (Fang et al., 1989). An *rbcS-3C* 3′ nontranslated region was used as the probe for quantitative determination of the GUS transcripts by S1 nuclease protection assays (Fluhr et al., 1986).

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