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# Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants

**(organ specificity/transgenic tobacco/site-specific mutagenesis)**

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**ABSTRACT** **The 35S promoter of cauliflower mosaic virus (CaMV) is able to confer high-level gene expression in most organs of transgenic plants. A cellular factor from pea and tobacco leaf tissue, which recognizes nucleotides in a tandemly repeated TGACG motif at the −75 region of this promoter, has been detected by DNase I footprinting and gel retardation assays. This factor is named activation sequence factor 1 (ASF-1). A cellular factor binding to the two TGACG motifs can also be detected in tobacco root extracts. Mutations at these motifs inhibit binding of ASF-1 to the 35S promoter *in vitro*. When examined in transgenic tobacco, these mutations cause a 50% drop in leaf expression of the 35S promoter. In addition, these same mutations attenuate stem and root expression of the 35S promoter about 5- to 10-fold when compared to the level of expression in leaf. In contrast, mutations at two adjacent CCAAT-box-like sequences have no dramatic effect on promoter activity *in vivo*. A 21-base-pair element containing the two TGACG motifs is sufficient for binding of ASF-1 *in vitro* when inserted in a green-tissue-specific promoter. *In vivo*, the insertion of an ASF-1 binding site caused high levels of expression in root. Thus, a single factor binding site that is defined by site-specific mutations is shown to be sufficient to alter the expression pattern of promoters *in vivo*.**

Cauliflower mosaic virus (CaMV) is a double-stranded DNA plant virus. It contains two promoters responsible for producing transcripts of 35S and 19S in infected plants (1). The 35S promoter is active in isolated protoplasts of monocots and dicots (2) and is also expressed in various organs of transgenic tobacco plants with no requirement for any viral protein. The high activity and constitutive expression of the 35S promoter provide a useful system to investigate cis-regulatory elements for plant gene transcription. Deletion analyses of the 35S promoter using protoplasts or transgenic tobacco as assay systems have been reported by several laboratories (3–6). A number of internal deletion studies have indicated that the region between −90 and −46 is important for the maximal expression of the 35S promoter (4–6). In addition, this same region has been shown to cause constitutive expression in maize cells when placed near the anaerobic response element of the maize alcohol dehydrogenase gene (7). Recently, our laboratory has reported that the 35S promoter, when deleted to −90, is expressed preferentially in the root of transgenic tobacco (8). To gain a more precise definition of the sequences responsible for these activities, we have used the technique of DNase I footprinting to delineate the binding site of plant cell factors to the 35S promoter. In the present report, we characterize one such factor by site-specific mutations of its binding site. Subsequently the function of the binding site for this factor was studied in the context of the 35S promoter and a heterologous promoter in transgenic tobacco. We show that this cis element is both necessary and sufficient for expression in root.

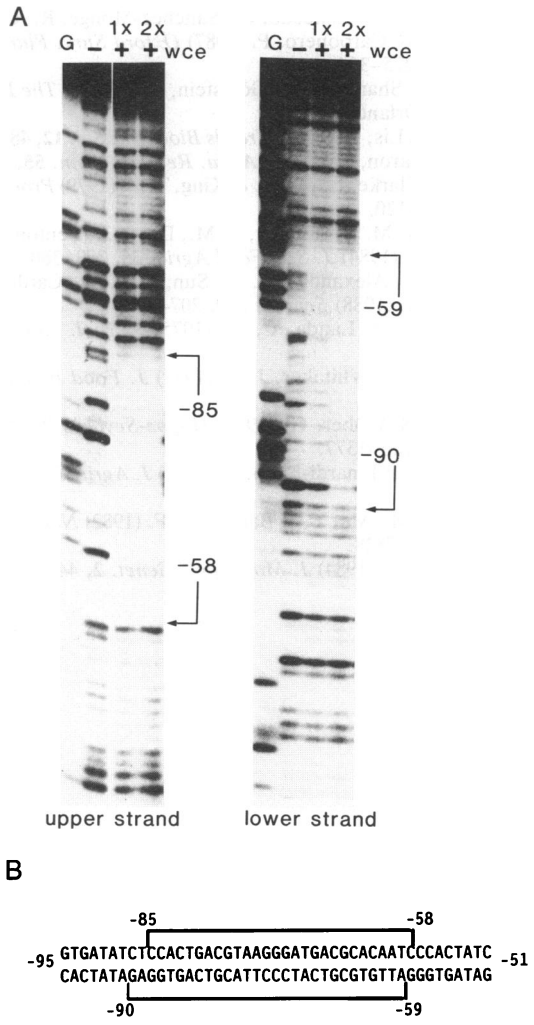


FIG. 1. Detection of a sequence-specific DNA binding factor from pea extract. (*A*) A probe extending from −130 to +2 of the 35S promoter was used to map the binding of a factor by DNase I footprinting. Data for the upper and lower strands are shown. Positions of the guanines (lanes G) were determined as described by Maxam and Gilbert (14). Control lanes without extract are shown by "−." When indicated with a "+," 2 μl (1×) or 4 μl (2×) of wce from pea seedlings was added. (*B*) The sequence of the region protected from DNase I digestion is shown.

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Abbreviations: CaMV, cauliflower mosaic virus; ASF-1, activation sequence factor 1; wce, whole-cell extract(s); CAT, chloramphenicol acetyltransferase.

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## MATERIALS AND METHODS

**Preparation of Extracts.** Pea whole-cell extract (wce) was prepared by the following method. Peas were grown for 7 days in the dark in order to deplete endogenous starch as well as to limit the synthesis of abundant photosynthetic enzymes, such as ribulose 1,5-bisphosphate carboxylase. Plants were harvested by cutting the hypocotyl 2.5–5 cm below the hook region. The tissues were rinsed with cold distilled water and then blended in cold buffer A [0.3 M sucrose/40 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/0.8 mM phenylmethylsulfonyl fluoride (PMSF)/0.01 mM benzamidine/0.05 mM ε-amino-*n*-caproic acid] and the homogenate was filtered through 1000-μm and 80-μm mesh filters. One-tenth volume of 5 M NaCl was added to the filtrate and, after gentle mixing, the mixture was centrifuged at 40,000 rpm in a Beckman Ti70 rotor for 1 hr. Solid ammonium sulfate was added to the supernatant solution to a final concentration of 0.3 g/ml. The precipitate was collected by centrifugation and then resuspended in buffer B (20 mM Hepes-KOH, pH 7.0/40 mM KCl/1 mM EDTA/10% glycerol/0.8 mM PMSF/0.5 mM dithiothreitol) to a final protein concentration of about 30 mg/ml. After dialysis against 2 liters of buffer B for 3 hr at 4°C, the extract was centrifuged at 10,000 rpm for 10 min before storage in small aliquots at −80°C until use. Nuclear extracts were prepared from 7-day-old green pea seedlings, mature tobacco leaves, or tobacco roots by the procedure described in Green *et al.* (9) except the Percoll gradient step was omitted.

**DNase I Footprinting and Gel Mobility Shift Assays.** DNase I footprinting was carried out by a modification of the method in Green *et al.* (9). Buffer B was used for the incubation, and the treatment with DNase I was stopped by the addition of 0.75 volume of a solution containing 5 mg of proteinase K per ml and 0.1 M EDTA. The mixture was then incubated for 20 min at 37°C before extraction with phenol:chloroform, 1:1 (vol/vol), and subsequent precipitation of the probe for sequencing gel analysis. The gel mobility shift assay was carried out as described by Mikami *et al.* (10) using a polyacrylamide/agarose composite gel.

**Plasmid Constructs and Transgenic Plant Analysis.** Mutations were constructed in the 35S promoter by synthesis of oligonucleotide pairs extending from the *Eco*RV site at −90 to the +2 position. These oligonucleotides were then ligated back to the −343 to −90 fragment to reconstitute the uninterrupted promoter. Probes containing four copies of the sequence from −82 to −62 of the wild type or *as-1c* promoter in a head to tail configuration were also synthesized. The tetramer binding sites were flanked by *Hin*dIII and *Xho* I linkers at the 5′ and 3′ ends, respectively, and subsequently cloned into a pEMBL 12 derivative with an *Xho* I site inserted into the *Bam*HI site. The probes used were labeled at the *Xho* I and *Hin*dIII sites by Klenow fill-in and subsequently purified by acrylamide gel electrophoresis and electroelution. For construct I, the binding site of activation sequence factor 1 (ASF-1), from −82 to −62 of the 35S promoter, was introduced into the pea *rbcS-3A* promoter by the insertion of synthetic oligonucleotide pairs into the *Bst*XI site at −55. In construct III, the ASF-1 binding site was created as site-specific mutations in the −109 to −89 region during the reconstruction of the entire −166 to −55 region of the *rbcS-3A* promoter by a series of complementary synthetic oligonucleotides. Constructs II and IV containing the *as-1c*

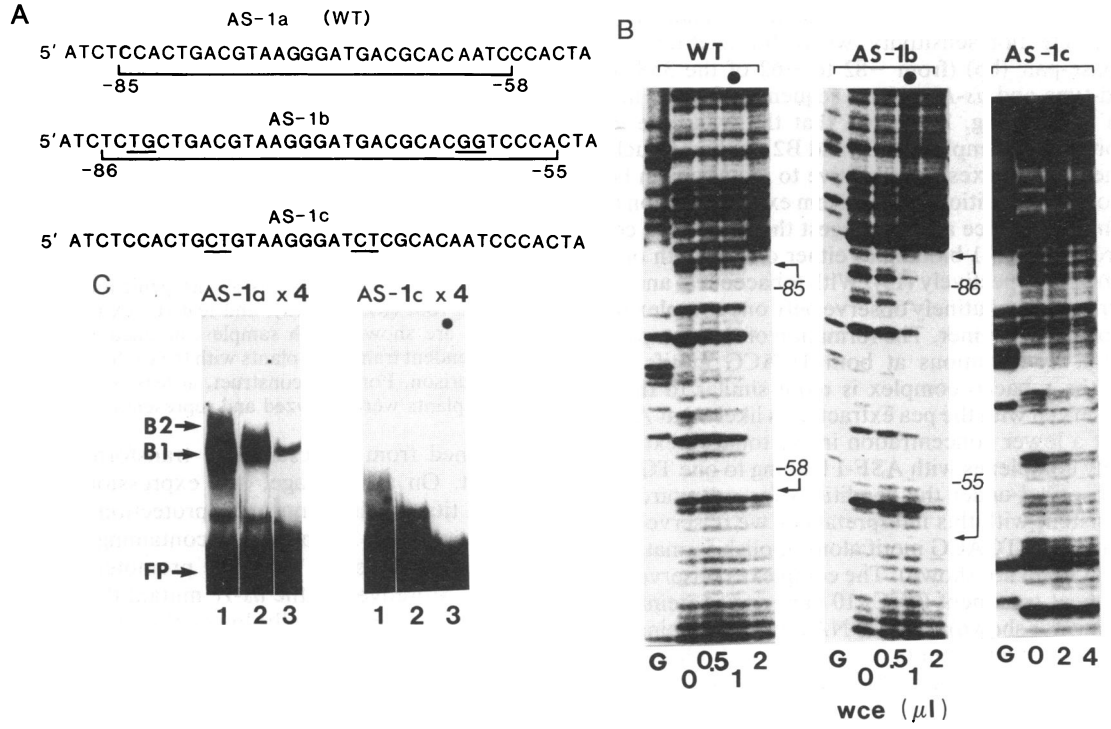


FIG. 2. Characterization of the ASF-1 binding site by site-specific mutations. (*A*) Mutations created within the binding site of ASF-1. The wild-type (WT) sequence of the 35S promoter (−89 to −52) is shown along with those of the mutated promoters *as-1b* and *as-1c*. The mutated bases are underlined and the footprinted areas are indicated by brackets under the WT and *as-1b* sequences. Only the upper strand of the DNA is shown. (*B*) The effects of the mutations were studied by extract titration with the DNase I footprinting assay. For the WT and *as-1b* probes, wce of 0.5, 1.0, and 2.0 μl were used. For the *as-1c* probe, 2 and 4 μl of wce were used. The lanes marked with ● show differences in the extent of protection between the WT and *as-1b* probes. (*C*) Gel mobility shift analysis of nuclear extracts from various sources. Extracts from crude nuclear preparations of pea leaves (lanes 1), tobacco leaves (lanes 2), and tobacco roots (lanes 3) were used in gel mobility shift assays with probes containing the tetramer of the WT (AS-la × 4) or the *as-1c* mutant sequence (AS-1c × 4). The positions of the free probe (FP) and the observed sequence-specific complexes B1 and B2 are indicated. Nuclear extracts from pea leaf (15 μg), tobacco leaf (20 μg), and tobacco root (4 μg) were added to a reaction mixture of 10 μl final volume.

mutant sequences were made in the same way as constructs I and III, respectively. Constructs were inserted into pMON505 or pMON200 derivatives and transferred into *Agrobacterium tumefaciens* by way of three-way mating, and the exconjugants were used for transformation of tobacco (cv. Xanthi) by the leaf disk method (11). Total RNA of root and leaf from independent transgenic plants was isolated and analyzed by 3′ S1 nuclease protection assay using the pea *rbcS-E9* 3′ probe (12). Chloramphenicol acetyltransferase (CAT) activities in plant organ extracts (10 μg of protein) were assayed as described (8, 13).

## RESULTS

Fig. 1 shows the detection of a factor in wce from pea that interacts specifically with sequences between −90 and −58 of the 35S promoter. Because this region has been implicated in the activation of upstream elements (4–7), we have named the binding site *as-1* (activation sequence 1) and the detected factor ASF-1. *as-1* contains two putative CCAAT-box-like sequences as well as two tandem TGACG motifs. To determine which of these sequence elements is important for binding of ASF-1, we created site-specific mutations in the respective motifs. The resultant mutant sequences (*as-1b* and *as-1c*) are shown in Fig. 2*A*. When examined for binding activity by DNase I footprinting, *as-1b* was found to bind ASF-1 with a slightly higher affinity than the wild-type *as-1* sequence, whereas *as-1c* was found not to bind ASF-1 to any significant extent (Fig. 2 *B* and *C*). In addition, competition experiments show that *as-1b*, but not *as-1c*, can compete with the wild-type promoter for ASF-1 binding (15). Although wce from pea provides a readily available source of plant cell extracts, we wanted to establish the existence of ASF-1 in tobacco since this plant is our system of *in vivo* analysis. To enhance our detection sensitivity, we synthesized tetramers of the 21-base-pair (bp) (from −82 to −62 of the 35S promoter) wild-type and *as-1c* mutant sequences for use in gel retardation assays. Fig. 2*C* shows that the wild-type *as-1* tetramer forms two complexes, B1 and B2, with pea nuclear extract. These complexes are sensitive to mutations in both TGACG motifs. In addition, results from extract titration and methylation interference assays suggest the B1 and B2 complexes represent ASF-1 binding to either one or both of the TGACG motifs, respectively (15). With tobacco leaf and root nuclear extracts, we routinely observe only one complex with the wild-type *as-1* tetramer. The formation of this complex is also inhibited by mutations at both TGACG motifs. The mobility of the tobacco complex is more similar to the B1 complex observed with the pea extract. It is likely that ASF-1 is present at a lower concentration in the tobacco extracts, and thus only complexes with ASF-1 binding to one TGACG motif are observed under the conditions for gel retardation assay. Consistent with this interpretation, we observed that mutations at either TGACG motif alone abolish formation of B2 but not B1 (data not shown). The complexes observed are sensitive to heat treatment (65°C, 10 min) and proteinase K digestion (data not shown). Thus, DNA binding proteins with similar sequence requirements are present in both pea and tobacco and, at least in tobacco, this activity is present in leaf as well as root cells.

To examine the function of ASF-1, we have inserted the 35S promoter (from −343 to +2) with either *as-1*, *as-1b*, or *as-1c* sequences in the vector shown in Fig. 3*A*. The reporter gene for promoter activity is the CAT coding region, and, as a reference, the wild-type 35S promoter (−941 to +8) fused to the β-glucuronidase (GUS) gene is inserted downstream in the pMON505 vector. Mature transgenic plants were obtained for each construct and the enzymatic activities of the CAT and GUS gene products were measured in root, stem, and leaf tissue from each transformant. Fig. 3 shows the data

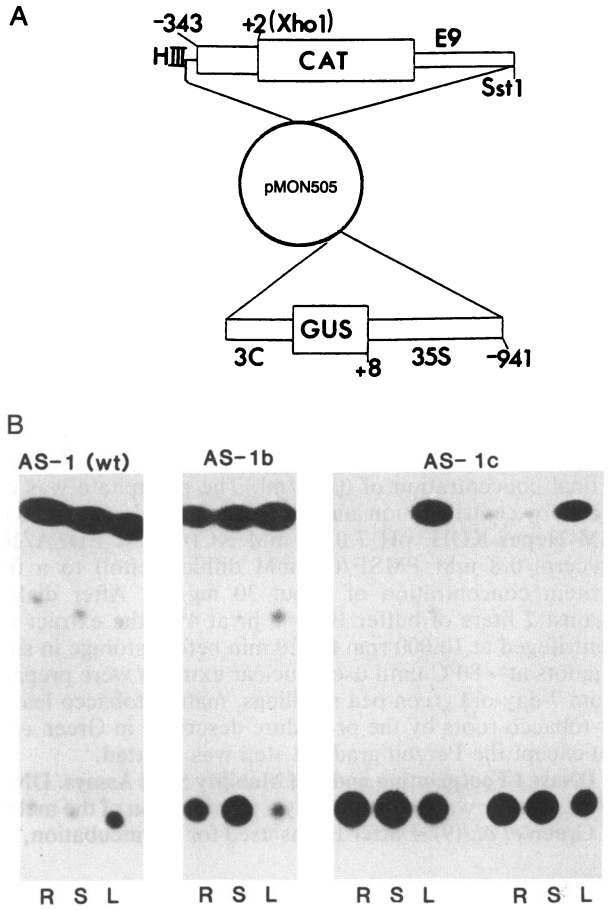


FIG. 3. Effects of binding site mutations on the expression of the 35S promoter *in vivo*. (*A*) The wild-type (WT), *as-1b*, and *as-1c* 35S promoters (−343 to +2) were fused 5′ to the bacterial CAT coding sequence followed by the pea *rbcS-E9* gene 3′ poly(A) sequence (6). In this pMON505 construct, the reference gene is composed of the 35S promoter (−941 to +8), the β-glucuronidase (GUS) coding sequence, and the *rbcS-3C* 3′ poly(A)region. (*B*) Organ specificity of the 35S promoter mutants. The test gene constructs shown in *A* with the WT, *as-1b*, and *as-1c* 35S promoters were assayed for their expression in various organs of transgenic tobacco. The CAT activities in root (R), stem (S), and leaf (L) extracts from independent plants are shown. Each sample contained 10 μg of protein. Two independent transgenic plants with the *as-1c* construct are shown for comparison. For each construct, at least seven independent transgenic plants were analyzed and representative results are shown.

obtained from representative transformants for each construct. On the average, leaf expression as quantitated by RNA titration in S1 nuclease protection assays is about 50% lower with the 35S promoter containing the *as-1c* mutations as compared to the wild-type promoter (E.L., unpublished data). Moreover, in the *as-1c* mutant the expression in stem and root is dramatically lower than that found in leaf. We found consistently that stem and root contain only 10–20% of the CAT activity in leaf among individual transformants with the *as-lc* 35S promoter. Fig. 3 shows data from two independent transformants to give an example of the variation. The promoter with *as-1b* shows a similar expression pattern to the wild type. In addition, a fluorimetric assay of GUS activities of the reference gene using the same extracts showed high activities, with different organs from individual plants showing differences of <2-fold (data not shown). Thus, binding of ASF-1 to the 35S promoter appears to be important for constitutive expression since mutations that interfere with factor binding *in vitro* more drastically attenuate *in vivo* promoter activity in stem and root relative to leaf.

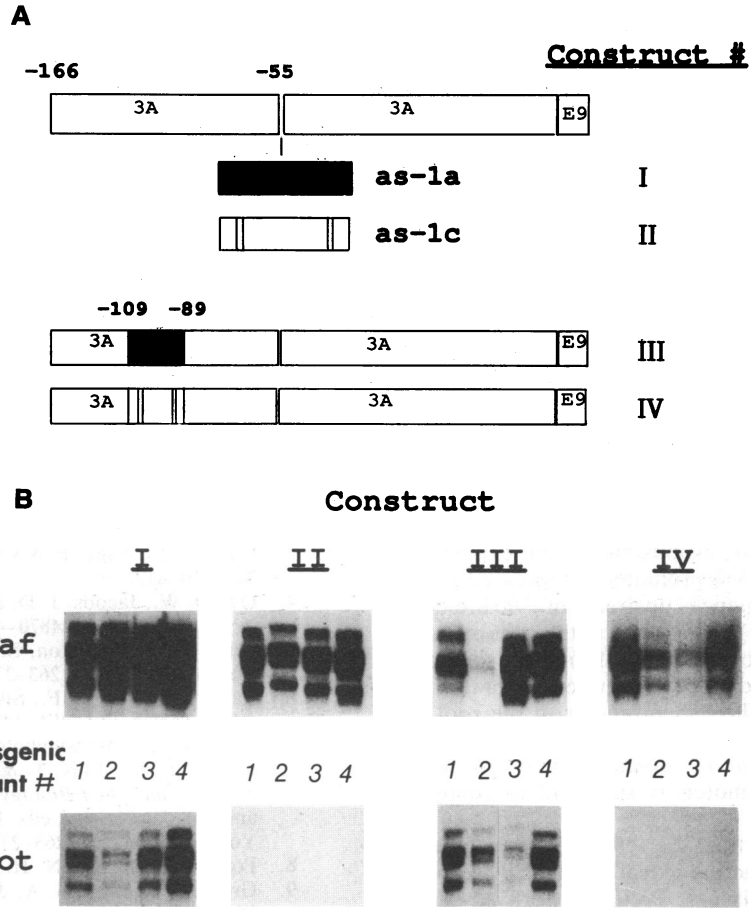


FIG. 4. Insertion of the ASF-1 binding site into the pea *rbcS-3A* promoter results in high expression in roots. (*A*) In construct I, the binding site of ASF-1 was introduced into the pea *rbcS-3A* promoter by the insertion of synthetic oligonucleotide pairs into the *Bst*XI site at −55. In construct III, the ASF-1 binding site was created as site-specific mutations in the −109 to −89 region. Constructs II and IV containing the *as-1c* mutant sequences were made in the same way as constructs I and III, respectively. The positions of the mutated or inserted sequences are indicated by the shaded (for *as-1a*) or striped (for *as-1c*) boxes. (*B*) Total RNAs of leaf and root from independent transgenic plants containing constructs I–IV were analyzed by 3′ S1 nuclease protection assays. The sequences shown in *A* were inserted into pMON200 and subsequently transferred into tobacco by way of *A. tumefaciens*. Roots and leaves from transgenic plants growing in Murashige–Skoog/agar containing kanamycin (100 μg/ml) and carbenicillin (500 μg/ml) were used for RNA preparation and 3′ S1 nuclease protection assay. Four individual transformants are shown for each construct. Each lane contained 10 μg of total RNA. The S1 signals show a cluster of protected fragments of about 230 nucleotides (6, 12, 16, 17).

To gain more direct evidence for the function of the ASF-1 binding site, we placed the 21-bp element (−82 to −62 of the 35S promoter) within the promoter for the gene encoding the small subunit of ribulose 1,5-bisphosphate carboxylase (*rbcS*), which is preferentially expressed in green tissues of transgenic tobacco (16, 17). We inserted the 21-bp element into the pea *rbcS-3A* promoter at −55 relative to the transcription start site (construct I in Fig. 4). As a control, we inserted the *as-1c* mutant in the identical position (construct II). In addition, to avoid moving the upstream regulatory sequences, such as box II and box III (9, 17), to a new location relative to the transcription start site, we introduced the *as-1a* and *as-1c* sequences into the −100 region of *rbcS-3A* by base-specific substitutions (constructs III and IV). The resultant constructs were transferred into tobacco, and total RNAs were prepared from leaf and root of individual transformants. Because our laboratory has previously characterized probes for the 3′ ends of pea *rbcS* genes (12), we quantitated the expression of the different constructs in transgenic tobacco by 3′ S1 nuclease assays with total RNA. Fig. 4 shows that insertion of *as-1*, but not the *as-1c* mutant, into the *rbcS-3A* promoter results in elevated expression in root. Based on the sensitivity of our S1 nuclease protection assay, we estimate the observed root expression in constructs I and III to be at least 20-fold higher as compared to that in constructs II and IV. In addition, the leaf expression of constructs I and III also appears to be about 2-fold higher than those of constructs II and IV, respectively. These results show that factors with the binding specificity of ASF-1 can act as positive elements for gene expression in root and leaf of transgenic tobacco.

## DISCUSSION

**Characterization of a Factor Binding to the CaMV 35S Promoter.** Our present work documents a cellular factor that interacts with the CaMV 35S promoter. This factor, ASF-1, binds to a region that has been implicated in modulating the quantitative (4–6) and qualitative (7) aspects of the expression of upstream elements. Since ASF-1 activity is present in both leaf and root of tobacco, the factor does not appear to be tissue specific. Furthermore, it appears to be conserved among pea and tobacco. By using methylation interference assays and site-specific mutagenesis (15), we concluded that the repeated TGACG sequence found in *as-1* is required for DNA–protein interaction. This factor binding site sequence shares homology with those for the mammalian factors such as ATF/CREB (cAMP-responsive element binding protein) (18) and JUN/AP-1 (19, 20). Interestingly, a single copy of this TGACG motif is also found in the promoter region of the

wheat histone H3 gene, which interacts with a factor designated HBP-1 (10). In fact, we found that the binding site for HBP-1 can compete with *as-1* for binding of ASF-1 in our extracts (E.L., unpublished data). Thus, HBP-1 is likely to be related, if not identical, to ASF-1. At present, the functional role of the HBP-1 binding site in the expression of the histone gene promoter is unknown. In the case of the 35S promoter, reported here, site-specific mutations at the two CCAAT-like sequences flanking the ASF-1 binding site demonstrated that they are not required for DNA–protein recognition *in vitro*. *In vivo*, these mutations do not cause significant changes in promoter activity in leaf, stem, and root. In contrast, mutations at the two TGACG motifs interfered with ASF-1 binding *in vitro* and preferentially attenuated root and stem expression of the 35S promoter *in vivo*.

**Role of ASF-1 in Root and Leaf Expression of the 35S Promoter in Tobacco.** Our data indicate that in the absence of a functional ASF-1 binding site at the −75 region, the 35S promoter becomes preferentially expressed in leaf relative to stem and root. This result suggests that, in addition to ASF-1, at least one other factor is responsible for leaf expression of the promoter. Since the 35S promoter is expressed predominantly in root when sequences upstream of −90 are deleted (8), it is likely that ASF-1 is involved in the root expression of this promoter. Moreover, results from our site-specific mutations of *as-1* also indicate the requirement of this factor for high expression of the 35S promoter in the root. This interpretation is supported by the gain-of-function experiment in which a single *as-1* sequence, when inserted in a green-tissue-specific promoter, is sufficient to confer root expression.

We find it intriguing that the −90 deletion of the 35S promoter is relatively inactive in leaf since ASF-1 is present in leaf as well as root tissues. Previous work has demonstrated that the *as-1* region can interact synergistically with upstream elements for high-level leaf expression (6, 7). Our results reported here show that site-specific mutations of *as-1* lead to a lower 35S promoter activity in leaf. Taken together, these data suggest that *as-1* is also important for leaf expression. The inability of the −90 derivative of the 35S promoter to express in leaf indicates at least one other factor that binds upstream of −90 is necessary for leaf expression. In contrast, a single *as-1* sequence appears to be sufficient for root expression. To explain this difference of dependence on upstream elements (−343 to −90) for root and leaf expression of the 35S promoter, we propose that the amount of active ASF-1 may be more limiting in leaf than in root. According to this model, a single *as-1* sequence is not sufficient to confer leaf expression. However, in the presence of upstream elements, ASF-1 binding *in vivo* can be stabilized by interactions with factors that bind to the upstream elements. Since we have not characterized the upstream factors of the 35S promoter at this time, we cannot rule out the possibility that some of these factors may also be limiting. However, it is evident that they can be active in the absence of ASF-1 binding to the *as-1* sequence (ref. 6 and this work). Thus, cooperative interaction among these factors, including ASF-1, can potentially decrease the dissociation constant for factor binding *in vivo* and thus overcome their concentration limitation in leaf cells. This hypothesis is consistent with the observed increase in leaf expression when the wild-type *as-1* sequence is inserted into the *rbcS-3A* promoter. In the root, the amount of functional ASF-1 may be sufficiently abundant so that there is less dependence on interaction with upstream factors.

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