Plant Nuclear Factor ASF-1 Binds to an Essential Region of the Nopaline Synthase Promoter*

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We have characterized a tobacco nuclear factor that binds to the −118 region of the nopaline synthase (nos) promoter from the Ti plasmid of Agrobacterium tumefaciens. The binding site for this factor, identified by DNase I footprinting, encompasses the region from −138 to −103 of the nos promoter. This region, which contains a potential Z-DNA-forming sequence, was previously shown to be essential for nos promoter activity in transgenic tobacco. A synthetic 21-bp sequence experimentally derived from the protected region (from −131 to −111), designated as nos-1, was sufficient for factor recognition in vitro. In transgenic tobacco, a tetramer of nos-1 can confer leaf and root expression when fused upstream of a truncated 35 S promoter from the cauliflower mosaic virus. Mutations at the two TGACG-like motifs in nos-1 abolish factor binding while preserving the potential for Z-DNA formation. A tetramer of the nos-1 mutant sequence has no significant activity above background when tested in transgenic tobacco. Competition experiments with activation sequence factor (ASF)-1 binding sites from the 35 S promoter of cauliflower mosaic virus (asm-1) and the wheat histone H3 promoter (hes-1) demonstrate that ASF-1 is the factor that binds to nos-1.

Agrobacterium tumefaciens infects its host through the transfer and subsequent integration of its T-DNA into the host genome. The T-DNA contains genes for plant hormone and opine synthesis. In the host nucleus, but not in the bacteria, the genes for opine synthesis are transcribed efficiently (Drummond et al., 1977; Joos et al., 1983). Thus, it is likely that promoters of these genes are able to utilize host nuclear factors for their expression. Among these promoters, the nopaline synthase (nos) promoter has been widely used to express the bacterial neomycin phosphotransferase II as a positive selection marker in plant transformation systems. Earlier workers have reported that the nos promoter is constitutively expressed (Herrera-Estrella et al., 1984; Simpson et al., 1986). However, more detailed studies by An et al. (1989) have shown that, in fact, it is developmentally regulated in transgenic tobacco. Thus, in transgenic tobacco, the nos promoter is expressed well in roots and mature leaves but not in young leaves. In contrast, in the same study the 35 S promoter from cauliflower mosaic virus (CaMV) was found to be essentially constitutive.

Early deletion analyses using Kalanchoe calli as an expression system suggested that sequences upstream of −88 of the nos promoter are dispensable for maximal activity (Shaw et al., 1984). However, more recent studies with tobacco calli and transgenic tobacco showed that several cis-elements upstream of the TATA box region are essential for promoter activity (Ebert et al., 1987; Ha and An, 1989). Using tobacco calli as the assay system, internal deletion studies showed that at least two regions (−97 to −63 and −119 to −101) of the nopaline synthase promoter are required for activity (Ebert et al., 1987). In transgenic tobacco, 5′-deletion analysis showed a major drop in activity at −130, and further deletion to −101 resulted in a complete loss of activity (Ha and An, 1989). This work thus suggests that sequences between −130 and −101 are important for nopaline synthase promoter function in transgenic tobacco. An alternating purine-pyrimidine stretch located between −123 and −114 has the potential to form Z-DNA. Ha and An (1989) have suggested that this region is involved in the temporal as well as the organ-specific expression of the nos promoter. In contrast to the detailed cis-element analysis, trans-acting factors that bind to sequences within the nos promoter have not been characterized.

In the present work, we have used the DNase I footprint technique to identify a factor binding site at the −118 region of the nos promoter. The binding activity can be detected in nuclear extracts from tobacco root and leaf. We showed that a tetramer of the 21-bp factor binding site (−131 to −111), designated as nos-1, can function as a promoter element in both leaf and root of transgenic tobacco. Site-specific mutations in nos-1 suggest that factor binding, but not Z-DNA formation potential, is correlated with the activity of the nos-1 tetramer in transgenic plants. By competition experiments, we have demonstrated that activation sequence factor (ASF)-1, a previously characterized plant factor (Lam et al., 1989; Katagiri et al., 1989; Fromm et al., 1989), is the one that binds to nos-1 in vitro.

**MATERIALS AND METHODS**

**Plasmids, Oligonucleotides, and Vectors—**Synthetic oligonucleotides were annealed and cloned into pEMBL 12 derivatives via the unique HindIII and XhoI sites. The cloned oligonucleotides were all sequenced before use. The vector X-GUS-46 was constructed from pMON506 by a similar method as the one reported for the X-GUS-90 vector (Beney and Chua, 1989). The −46 to +8 sequence of the 35 S promoter from CaMV was placed upstream of the β-glucuronidase (GUS) coding sequence. Unique HindIII and XhoI restriction sites are available immediately 5′ to the 35 S promoter sequence in order to facilitate the insertion of our cloned oligonucleotides. Details of the cloning of ASF-1 binding sites into the rbcS-3A promoter have been described (Lam et al., 1989).

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1The abbreviations used are: CaMV, cauliflower mosaic virus; ASF, activation sequence factor; bp, base pair(s); TRM, TGACG-related motifs.
been reported previously (Lam et al., 1989; Gilmartin and Chua, 1989). The tetramers of the various wild-type and mutant binding sites were synthesized as complementary oligonucleotides with four direct repeating units of 21 bp. Unique HindIII and XhoI restriction sites were placed at the 5' and 3' ends, respectively, of these 84-bp units.

Characterization of Protein-DNA Interactions—Isolation of pea whole cell extracts, tobacco root and leaf nuclear extracts, gel retardation assay, and DNase I footprinting were carried out as described previously (Lam et al., 1989; Green et al., 1989).

Transformation of Tobacco and Analysis of GUS Gene Product—Nicotiana tabacum (cv. SR1) was used for transformation with the pMON505 derivatives by the leaf disc method (Fraley et al., 1986). Typically, 5-10 independent transgenic tobacco plants were analyzed for each construct. GUS activity in leaf and root extracts was measured as described previously (Benfey et al., 1989). Two time points for each sample were measured in order to ensure linearity of activity.

RESULTS

Sequence-specific DNA Binding Factors Interacting with the nos Promoter in Vitro—Our goal was to characterize nuclear factors from tobacco extracts that interact with the regions reported to be important for the nos promoter activity (Ha and An, 1989). The promoter region around −112 is especially interesting since it contains a TGACG motif in the reverse orientation relative to the transcription start site. Recent studies from our laboratory have indicated that TGACG-related motifs are involved in the binding of a tobacco nuclear factor, ASF-1, to the 3S promoter of CaMV and the wheat histone H3 promoter (Katagiri et al., 1989; Lam et al., 1989, 1990; Lam and Chua, 1989). In order to characterize factors that may interact with this region of the nos promoter, we synthesized an oligonucleotide pair extending from −150 to −91 of the nos promoter and used it for DNase I footprinting. Fig. 1 shows that whole cell extracts from pea and nuclear extracts from tobacco give identical protection of a region between −138 and −103 of the nos promoter. As indicated in Fig. 1, the TGACG motif and a related sequence, TGAGC, further upstream are located within the protected region.

To compare the binding of factors from different organs of tobacco and to demonstrate the involvement of the two putative TGACG-like motifs in factor binding, we synthesized a wild-type and a mutant tetramer of the 21-bp sequence from −131 to −111 of the nos promoter. The mutant differs from the wild type in 4 of the 21 bp, with the mutations at the two TGACG-like motifs (Fig. 2). The substituted bases were chosen to preserve the purine/pyrimidine context of the promoter sequence in order not to perturb the potential for Z-DNA formation. Fig. 2 shows that tobacco nuclear extracts from leaf give at least two complexes with the nos-1 tetramer probe. Only the B1 complex was seen at a lower extract concentration (data not shown). The mutations introduced at the two TGACG-like motifs abolish formation of these complexes. Tobacco root nuclear extracts, at about a 5-fold lower protein concentration, give a complex similar to the B1 complex observed with the leaf extracts. In this case, the complex formation is also sensitive to the mutations. These results suggest that tobacco root and leaf contain factors that can bind to nos-1 with similar sequence specificity. Our data thus indicate that sequences in at least one of the two TGACG-like motifs are involved in protein-DNA interaction.

Functional Characterization of nos-1 Tetramer in Transgenic Tobacco—To examine the functional properties of the nos-1 element, we fused the tetramer of the wild-type and mutant nos-1 sequences to an expression vector containing the bacterial β-glucuronidase (GUS) coding sequence as a reporter gene. A truncated derivative of the CaMV 35S promoter (−46 to +8) was used as the TATA box element. Table I shows the GUS activity of soluble extracts from root and leaf of transgenic tobacco plants with these constructs. In the absence of any upstream elements, the −46 derivative
of the 35 S promoter (X-GUS-46) does not show any significant activity above background. When a tetramer of the nos-1 sequence was fused directly upstream of the −46 position (construct 4N1-46), high activity was obtained in both leaf and root of tobacco. Interestingly, the activity in root is significantly higher than that found in leaf. In both organs, the mutant nos-1 tetramer shows much lower activity. Immunohistochemical localization of the GUS gene product shows that the tetramer of nos-1 can confer expression in most cell types in leaf and root (data not shown). These results demonstrate a direct correlation between factor binding to nos-1 in vitro and the ability to confer positive transcriptional activity in vivo.

Comparative Analysis of Three TGACG-containing Factor Binding Sites for the Tobacco Nuclear Factors ASF-1 and HSBF—We have previously reported that tobacco ASF-1 binds to activation sequence (as) −1, a 21-bp sequence at the −75 region of the 35 S promoter from CaMV, which contains a tandem TGACG motif (Lam et al., 1989, 1990). We have also found that the binding site of the wheat nuclear factor HBP-1 (Mikami et al., 1987), which also contains a TGACG motif, can compete with as-1 for ASF-1 binding (Lam and Chua, 1989). As we have noted above, the nos-1 sequence also contains two TGACG-like motifs. In addition, the formation of the two complexes, B1 and B2, is very similar to the previous observation with the as-1 tetramer (Lam et al., 1989, 1990). Thus, we examined the possibility that ASF-1 is the factor that binds to nos-1 as well. Complementary oligonucleotides corresponding to a 21-bp sequence from the wheat histone H3 (hex-1), the CaMV 35 S (as-1), and the nopaline synthase (nos-1) promoters were synthesized. The sequences between −109 and −89 of the pea rbs-3A promoter were replaced by these sequences as described previously (Gilmartin and Chua, 1989). Mutant sequences with mutations in the TGACG motifs of these three elements were also synthesized and cloned by similar procedures. The sequences of these oligonucleotides are shown in Fig. 2B. The resulting chimeric promoter fragments were used as binding site probes in gel retardation assays with nuclear extracts from tobacco leaf. Nonlabeled fragments containing these binding sites were used as competitors. Fig. 3A shows that tobacco ASF-1 binds not only to as-1 and hex-1 but to nos-1 as well. This complex observed with the three probes can be competed by the wild type but not by the mutant competitor fragments of the other two binding sites. However, in contrast to the other two elements, hex-1 also forms another specific DNA-protein complex (marked as HSBF, Fig. 3A) with a faster mobility as compared with the complex with ASF-1. Competition assays show that this hex-1-specific complex is competed only by hex-1 wild type sequence but not by as-1, nos-1, or the corresponding mutant sequences shown in Fig. 3B. Together, these results show that in tobacco nuclear extracts there are at least two distinct DNA binding factors, ASF-1 and a hex-1-specific binding factor. We designate this latter binding activity HSBF.

**DISCUSSION**

**Comparative Analysis of Different Binding Sites for ASF-1 in Vitro**—In this work we have presented evidence that the tobacco nuclear factor ASF-1 can bind to three different
sequences originated from three phylogenetically diverse sources. Fig. 4 shows the binding sites used in our experiments, and sequences that have been shown to compete for ASF-1 binding are bracketed. In addition to the wheat histone gene promoter, the CaMV 35 S promoter, and the nos promoter from the T-DNA of A. tumefaciens, we have also included sequences from the octopine synthase (ocs) promoter as well. The ocs promoter originates from the T-DNA of the octopine type of A. tumefaciens, and the promoter region shown in Fig. 4 includes the 16-bp palindromic sequence previously shown to function as a positive transcriptional element in plant cells (Ellis et al., 1987). Recently, our laboratory has also demonstrated that this palindromic sequence can interact specifically with ASF-1 in vitro, and it exhibits a similar pattern of expression in transgenic tobacco as the -90 derivative of the 35 S promoter (Fromm et al., 1989). In all of these four binding sites, mutations in the TGACG-related motifs (TRM) abolish ASF-1 binding. In addition, at least for the CaMV 35 S promoter, the nos promoter, and the ocs promoter, the binding site sequences for ASF-1 have been shown to be important for promoter function in transgenic tobacco (Lam et al., 1989; Ha and An, 1989; Ellis et al., 1987). It is interesting that all of these three promoters of different origins, as suggested by their different sequences and arrangements of the TRM, have apparently selected ASF-1 as a critical factor for their function.

A comparison of the four ASF-1 binding sites reveals two interesting features. First, the three binding sites for ASF-1 of non-plant origin are comprised of two TRM, designated as the "dimeric" type, whereas the plant histone gene promoter binding site contains only a single copy of TGACG, designated as the "monomeric" type. Second, the arrangement and the distance between the TRM with respect to the TATA box as well as to each other do not appear to be highly conserved in the dimeric type ASF-1 binding sites.

With respect to the distinction between the dimeric and monomeric types of the ASF-1 binding site, it is important to point out that the hex-1 sequence can bind at least two nuclear factors with distinct sequence specificities. Thus, in addition to ASF-1, hex-1 can also interact with HSBF which does not bind to the dimeric ASF-1 binding sites. This observation is correlated with our recent cloning data which demonstrated the presence of two distinct classes of DNA binding proteins in tobacco (Katagiri et al., 1989). One class of DNA binding proteins, designated TGA1b, binds to both the dimeric and monomeric ASF-1 binding sites whereas the other class of DNA binding protein, designated TGA1a, binds significantly to the monomeric site only. Comparison of the sequence specificity between proteins encoded by these cDNA clones and tobacco nuclear factors suggests that TGA1a and TGA1b are likely to be components of the activities referred to as ASF-1 and HSBF, respectively. In this regard, we note that HBP-1, a cloned wheat hex-1 binding factor (Tabata et al., 1989), was shown recently to bind to the dimeric ASF-1 binding sites (Mikami et al., 1989). The functional properties of the hex-1 sequence remain to be determined.

The difference among the dimeric ASF-1 binding sites in spacing and orientation between the two TRM is intriguing. The number of base pairs between the two motifs varies between 4 (ocs promoter) and 10 (nos promoter). The orientation of the TRM exists as head to tail (35 S promoter), head to head (ocs promoter), and tail to tail (nos promoter). The motif also varies as a direct repeat of TGACG (35 S promoter), a TGACG and TGAGC imperfect palindrome (nos promoter), and a TTACG palindrome (ocs promoter). Single base mutations of the TRM in the ocs promoter suggested that mutations in one of the two motifs do not affect formation of the B1 complex observed in gel retardation assays (Singh et al., 1989). However, the B2 complex can be severely attenuated by single base mutations within one of the two TTACG motifs. We have observed similar effects of single site mutations in the as-1 sequence of the 35 S promoter. These data thus suggest strongly that ASF-1 can bind to an individual TRM in the dimeric binding sites. The B1 and B2 complexes observed in gel retardation assays are likely to represent ASF-1 binding to one and both TRM, respectively. Methylation interference studies of the B1 and B2 complexes with the as-1 sequence as the binding site support this conclusion (Lam et al., 1990). In protoplast assays, single base mutations in the TRM that abolish B2 complex formation were found to drastically lower activity (Singh et al., 1989). These results suggest that functional activity of the dimeric binding sites requires both TRM.

Functional Characterization of ASF-1 Binding Sites—We have previously demonstrated that a single copy of as-1 can activate the expression of the rbcS-3A promoter in root (Lam et al., 1989) and a single copy of as-1 alone can confer root-specific expression (Benfey et al., 1989). Moreover, the 16-bp palindromic sequence in the ocs promoter shows a similar expression pattern as as-1 in transgenic tobacco (Fromm et al., 1989). In this regard, we note that in the deletion study of Ha and An (1989), the -130 derivative of the nos promoter shows expression predominantly in root. A single copy of the nos-1 element is present at the 5' end of this nos promoter derivative. Taken together, these data suggest that a single copy of the "dimeric" binding site for ASF-1 confers preferential expression in root. In the present work, we have fused a tetramer of nos-1 to the -46 derivative of the 35 S promoter, which contains most likely only the TATA element. This chimeric construct gives high expression levels in both leaf and root, although root expression is consistently higher. Recently, we have found that a tetramer of as-1 can also confer leaf and root expression in transgenic tobacco. These results help to resolve the apparent paradox that although ASF-1 is present in leaf as well as in root, a single copy of the binding site confers expression predominantly in root. We have previously proposed that the functional form of ASF-1 may be more limiting in leaf than in root (Lam et al., 1989). The data presented here show that by increasing the number of ASF-1 binding sites we can overcome this factor limitation.

\[ E. Lam. \text{ unpublished data.} \]
Furthermore, analysis of the mRNA levels of the putative clone for ASP-1 showed that expression in root is about 10 times higher than in leaf of tobacco (Katagiri et al., 1989). These results are consistent with the proposal that the apparent root-specific expression of the −90 derivative of the 35S promoter and the −130 derivative of the nos promoter is due to the preferential expression of the ASP-1 factor in the root. An analogous picture has emerged in the recent analysis of the expression of the hunchback gene promoters and its regulatory factor bicoid in Drosophila (Struhl et al., 1989).

After the completion of this work, Mikami et al. (1989) reported similar findings that the nos-1, as-1, and hex-1 sequences bind to the same factor, HBP-1, in wheat and sunflower nuclear extracts. Thus, HBP-1 and ASF-1 have similar sequence specificity and interact with the monomeric sunflower nuclear extracts. However, we note that the sequence of wheat HBP-1 (Tabata et al., 1989) has no homology with those of the tobacco ASF-1 and HSBF (Katagiri et al., 1989) except at their putative DNA binding domains. Thus, the degree of similarity in the function and regulation among these factors remains to be determined. In addition, Bouchez et al. (1989) also reported that the maize factor OCSTF can bind to the as-1 and nos-1 sequences. Site-specific mutations by these workers have shown that the binding of the OCSTF to the dimeric type of binding sites is correlated with their function in Nicotiana plumbaginifolia cell culture protoplasts. In their system, a single copy of the dimeric type binding site is sufficient to confer expression. However, the activity of the binding sites was not tested in transgenic plants, and the chimeric promoter used was a maize alcohol dehydrogenase promoter truncated at −140. Thus, whether these dimeric type binding sites can independently confer expression in transgenic plant tissues was not addressed.

The functional characterization of the different dimeric ASF-1 binding sites in our laboratory has shown a remarkable similarity in their expression characteristics in transgenic plants (Lam et al., 1989; Benfey et al., 1989; Fromm et al., 1989; this work). All these sequences contain TRM and bind ASF-1. In the cases examined so far, mutations in the TRM attenuate ASF-1 binding in vitro and affect severely the activity of the sequence in transgenic tobacco. In the case of the nos-1 sequence, we show in the present work that the ability to form Z-DNA does not correlate with ASF-1 binding in vitro or the activity of this sequence in transgenic tobacco. These results provide strong evidence that the ASF-1 binding activity that we have characterized in vitro is responsible for the activity of the dimeric-type binding sites in transgenic tobacco.

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