An Octopine Synthase Enhancer Element Directs Tissue-Specific Expression and Binds ASF-1, a Factor from Tobacco Nuclear Extracts

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We have investigated the expression pattern conferred by a cis-regulatory element (−212 to −154) from the upstream region of the octopine synthase (ocs) gene in transgenic tobacco plants. Analysis of β-glucuronidase expression driven by the ocs regulatory element revealed a pattern that is tissue-specific and developmentally regulated. In young seedlings, expression is confined primarily to root tips. In older seedlings, expression is stronger and becomes apparent also in the shoot apex. Insertion of a 16-base pair palindromic sequence (−193 to −178), which is included in the regulatory element, into an rbcS promoter results in the expression of rbcS in roots. The 16-base pair palindrome binds activation sequence factor (ASF)-1, a factor from tobacco nuclear extracts that interacts with the sequence between −83 to −63, designated as activation sequence (as)-1, of the cauliflower mosaic virus 35S promoter [Lam et al. (1989). Proc. Natl. Acad. Sci. USA 86, in press]. The in vivo expression patterns and in vitro binding properties of the ocs palindromic sequence are remarkably similar to those of the as-1 element of the cauliflower mosaic virus 35S promoter. These results suggest the involvement of ASF-1 in the transcriptional regulation of the ocs promoter and the 35S promoter.

INTRODUCTION

The octopine synthase gene (ocs) of the Ti-plasmid of Agrobacterium tumefaciens encodes the enzyme involved in octopine biosynthesis from arginine and pyruvate in crown gall tissues (reviewed by Tempe and Goldman, 1982). The ocs gene is not expressed in the bacterium, but it contains all the signals necessary for expression in plants (Koncz et al., 1983). Analysis of plants transformed with the T-DNA of the Ti-plasmid revealed octopine synthase activity in leaves, stems, and roots (Otten et al., 1981). Based on this result, it was assumed that ocs is not regulated by environmental or developmental factors, although Otten et al. (1981) detected higher activities in roots than in other plant organs.

Promoter analysis by Koncz et al. (1983) suggested that a sequence of 125 bp of the ocs 5’ region (−294 to −170) is important for its own expression in plant tumors. Similarly, Leisner and Gelvin (1988) found that the region between −222 and −177 is essential for the activation of the ocs promoter in transformed calli. Detailed analysis of this upstream region revealed that a 16-bp palindrome (−193 to −178) can activate transcription of a heterologous promoter in protoplasts (Ellis et al., 1987). However, in spite of these detailed analyses of the ocs promoter in transformed calli and in protoplasts, the function of the ocs palindrome sequence has not been assessed in the context of a heterologous promoter in transgenic plants. It was not known whether the ocs palindrome would direct tissue-specific expression and whether the expression pattern would change during development.

In this study we have focused on the ocs palindrome enhancer sequence. We show that the ocs enhancer, when fused to the 35S TATA box (−46 to +8), can confer expression in the root tip of young and older seedlings as well as in the shoot apex of the older seedlings. Moreover, insertion of the ocs palidrome into the BstXI site (position −55) of the rbcS-3A promoter causes rbcS-3A to be expressed in roots. By gel shift assays and competition experiments, we have found that the ocs enhancer binds activation sequence factor (ASF)-1, a factor that interacts with the activation sequence (as)-1 element (−83 to −63) of the cauliflower mosaic virus (CaMV) 35S promoter (Lam et al., 1989). Our results suggest that transcription of the ocs and the CaMV 35S genes is regulated by at least one common trans-acting factor.
RESULTS

Tissue-Specific Expression of a GUS Reporter Gene Driven by ocs Enhancer Sequences

Octopine synthase promoter sequences were cloned into the X-GUS-46 vector shown in Figure 1A. This vector contains a 35S promoter fragment (−46 to +8), spanning the TATA box region, fused to the β-glucuronidase (GUS) coding sequence. Because previous studies have shown that 5’ deletions of the ocs promoter up to −206 and 3’ deletions up to −156 have little effect on the activity of the enhancer in protoplasts (Ellis et al., 1987), we first tested the ability of a 59-bp ocs promoter fragment containing 5’ sequences between −212 and −154 (59ocs, Figure 1B) to activate the 35S TATA box of the X-GUS-46 vector in transgenic plants. Primary transformants with this construct were analyzed for GUS expression. As a negative control, we used transgenic seedlings containing the X-GUS-46 vector. Histochemical analysis of nine independent transformants revealed that 59ocs directed GUS expression specifically in root tips (not shown) but not in leaves or stems. Histochemical analysis of transgenic plants containing the X-GUS-46 vector sequence, without ocs sequences, showed no GUS activity in roots, stems, and leaves.

The 59ocs (−212 to −154) contains a 16-bp palindrome (−193 to −178), which has been shown by Ellis et al. (1987) to act as a transcriptional enhancer in protoplast transient expression assays. To delineate the cis-element that activates the 35S TATA box in transgenic plants, we synthesized a 21-bp DNA fragment (−193 to −173) containing the 16-bp palindromic sequence (−193 to −178) and 5 bp of 3’ sequence, plus linker sequences (21ocs-wt, Figure 1C). As a negative control, we synthesized a mutant derivative of the same size (21ocs-mu, Figure 1C), which has been shown by Ellis et al. (1987) to be inactive in protoplasts. Both the 21ocs-wt and 21ocs-mu were ligated to vector X-GUS-46, and the constructs were transferred into tobacco plants as described in Methods.

Analysis of six primary transformants with the 21ocs-wt showed the same tissue-specific expression pattern as in transgenic plants with 59ocs, although the expression level was weaker with the shorter sequence. The 21ocs-mu construct did not give any detectable GUS expression in 17 independent transformants.

To study possible changes in patterns of expression during development, we analyzed transgenic F1 seedlings containing the three constructs described above. Transgenic seedlings containing either domain A (−90 to +8) or domain B (−343 to −90) of the CaMV 35S promoter fused to the GUS coding sequence were used as controls. Each of these domains was shown to confer a different developmentally regulated pattern of tissue-specific expression (Benfey, Ren, and Chua, 1989). Figure 2 shows the GUS expression profiles in transgenic F1 seedlings. Domain A conferred expression mainly in root tips of young seedlings (6 days old) (Figure 2E), and the expression was stronger in older seedlings (i.e., 17 days old) (Figure 2K). Transgenic seedlings with strong root expression also showed expression at the shoot apex (Figure 2K). By contrast, domain B mainly conferred expression in cotyledons and leaves (Fig-

Figure 1. Constructs Containing Octopine Synthase Enhancer Sequences Controlling GUS Expression in Transgenic Plants.

(A) Vector X-GUS-46. A DNA fragment of the CaMV 35S promoter (−46 to +8), containing the TATA box region, was ligated to the GUS coding sequence, with a 3’ end of the pea rbcS-3C gene (P. Benfey, unpublished data). This vector is similar to the vector X-GUS-90 (Benfey and Chua, 1989) except that in X-GUS-46, the CaMV 35S promoter sequence is from −46 to +8. The octopine synthase promoter sequences shown in (B) and (C) were ligated to the X-GUS-46 vector, 5’ to the 35S promoter sequence.

(B) A 59-bp DNA fragment (59ocs) of the octopine synthase promoter (−212 to −154) that contains the 16-bp palindromic sequence (−193 to −178) (arrows).

(C) A 21-bp DNA fragment (21ocs-wt) of the octopine synthase promoter (−193 to −173) that contains the 16-bp palindromic sequence (−193 to −178) (arrows) and a mutant sequence (21ocs-mu) of the same size that has four nucleotide changes relative to the 21ocs-wt sequence. Detailed nucleotide sequences of the 21ocs-wt and 21ocs-mu fragments and linker sequences for cloning these fragments into the X-GUS-46 vector are given in Methods.
Tissue-specific expression of GUS was also apparent when ocs enhancer sequences were present upstream of the 35S TATA box (−46 to +8) fused to the GUS coding sequence. Both 59ocs (Figures 2A and 2D) and 21ocs-wt (Figure 2B) directed GUS expression in root tips, with the former giving higher expression levels. With both constructs, expression was stronger in older seedlings (17 days versus 6 days; Figures 2G and 2H and Figures 2A and 2B, respectively). Moreover, transgenic seedlings that showed high GUS expression levels in roots (i.e., 17-day-old seedlings with 59ocs) also displayed GUS activity in the shoot apex (Figures 2G and 2J). No expression was detected in seedlings containing the 21ocs-mu construct (Figures 2C and 2I).

The expression pattern conferred by the ocs enhancer sequences (59ocs and 21ocs-wt) is remarkably similar to that observed with domain A of the CaMV 35S promoter. By contrast, domain B gave a different expression pattern (Benfey, Ren, and Chua, 1989).

### The 16-bp ocs Palindrome Confers Expression of rbcS in Roots

So far our results demonstrate that 59ocs (−212 to −154) and 21ocs-wt (−193 to −173) can activate the CaMV 35S TATA box (−46 to +8) and direct expression mainly in root tips. To investigate whether the 16-bp ocs palindrome alone can activate a heterologous promoter in transgenic plants, we introduced the 16-bp ocs palindrome, or the mutant sequence, into the BstXI site at position −55 of the pea rbcS-3A promoter (rbcS/ocs-wt and rbcS/ocs-mu, respectively), as described in Methods and shown in Figure 3A. Transgenic tobacco plants harboring these constructs were tested for the expression of the pea rbcS-3A transgene by S1 analysis of total RNA. Figure 3B shows that the 16-bp palindrome was sufficient to confer high levels of rbcS-3A expression in roots, whereas the expression of rbcS-3A with the mutant sequence was at least 10-fold lower in roots.

### The 16-bp ocs Palindrome Binds ASF-1 from Tobacco Nuclear Extracts and TGA1α, a Cloned DNA-Binding Protein

The patterns of tissue-specific expression conferred by the ocs element and by domain A of the 35S CaMV enhancer suggest that a common factor is involved in transcriptional regulation of the CaMV 35S and the ocs genes. To test this possibility, gel shift analyses were performed with tobacco extracts and wild-type and mutant ocs sequences as probes. Katagiri, Lam, and Chua (1989) have shown that a tobacco factor, designated as ASF-1, binds to the TGACG-containing sequences: as-1 (−83 to −63 of the CaMV 35S promoter; Lam et al., 1989), Hex1 (−180 to −160 of the wheat histone H3 promoter; Mikami et al., 1987), and nos-1 (−131 to −111 of the nopaline synthase promoter; An et al., 1986). Figure 4A shows that the same factor binds to the ocs palindrome. When the ocs wild-type sequence was used as a probe, a slow migrating band was apparent on the gel (arrow). The formation of this DNA/protein complex was competed by a 400-fold molar excess of wild-type ocs, as-1, Hex1, and nos-1 sequences but not by the corresponding mutant sequences. In a reciprocal experiment, where as-1 was used as probe, the formation of the ASF-1/DNA complex was competed by a 400-fold molar excess of the wild-type ocs palindromic sequence but not the mutant sequence (data not shown).

Recently, a cDNA encoding a protein factor (TGA1α) that specifically binds as-1, as well as other TGACG-containing cis elements, has been isolated in our laboratory (Katagiri, Lam, and Chua, 1989). The TGA1α protein is likely to be ASF-1, or a major component of ASF-1. We have prepared extracts from Escherichia coli overexpressing TGA1α and used them in gel mobility shift assays. Figure 4B shows that this extract binds strongly to ocs-wt but only weakly to ocs-mu. These results reinforce the identification of TGA1α as ASF-1.

### DISCUSSION

Dissection of 5′ upstream sequences of the ocs promoter has been carried out previously by several laboratories. In these studies, the activities of deletion mutants and/or chimeric constructs were measured either in protoplasts by transient expression assays (Ellis et al., 1987) or in transformed calli (Leisner and Gelvin, 1988). Two questions emerged in attempting to extrapolate the results of these studies to the regulation of ocs in plants. First, it was not known whether ocs sequences that are active in protoplasts and calli would retain their activities in the whole plant context. Second, the expression patterns of these elements in terms of their organ or tissue specificity were not addressed. To address these questions, we have assessed the activities of ocs 5′ sequences in transgenic tobacco plants.

In this paper we have shown that a 59-bp fragment (−212 to −154) from the ocs promoter, spanning a region previously shown to be active in protoplasts (Ellis et al., 1987), can indeed activate transcription of a heterologous TATA box in transgenic plants. Moreover, the fragment confers a striking degree of tissue-specific expression, expression that is detected principally in cells of the root tip as well as in cells of the shoot apex, with very little activity elsewhere in the plant. A 21-bp element (−193 to
Octopine Synthase Enhancer Element

Figure 3. The ocs Enhancer Confers Root Expression of rbcS.
(A) ocs enhancer sequences were introduced into the promoter of the pea rbcS-3A gene as detailed in Methods.
(B) S1 analyses of 10 μg of RNA from roots (R) and leaves (L) of transgenic tobacco plants containing wild-type rbcS-3A (rbcS), rbcS plus the ocs palindrome (rbcS/ocs-wt; eight independent transgenic plants analyzed), and rbcS plus the mutant sequence (rbcS/ocs-mu; seven independent transgenic plants analyzed) were carried out using an rbcS-E9 3' probe as described in Methods.

−173) (21ocs-wt) that resides within the 59ocs fragment directs a similar expression profile, except that the activities are, in general, lower. On the other hand, no activity of GUS was detected by histochemical analysis when a mutant derivative of the 21-bp element (21ocs-mu) was used. The 21ocs-wt element contains a 16-bp palindrome that has previously been shown to act as a transcriptional enhancer in protoplasts (Ellis et al., 1987; Singh et al., 1989). We have shown here that insertion of the 16-bp enhancer into the rbcS-3A promoter is sufficient to confer expression of rbcS-3A in roots.

A comparison of the expression patterns conferred by the 59ocs and 21ocs-wt sequences with that conferred by domain A of the 35S CaMV promoter revealed a striking similarity. A 21-bp cis-element, designated as as-1, has previously been found to be responsible for the activity of domain A in plants (Lam et al., 1989). The similarity in expression profiles prompted us to investigate whether the 16-bp ocs palindrome and as-1 interact with the same nuclear factor. By gel shift assays and competition experiments, we have shown that, indeed, the ocs palindrome binds to ASF-1 as well as to TGA1α. These two factors have been shown to interact with the two tandem TGACG motifs of as-1 of the CaMV 35S promoter. Figure 5A shows that the 16-bp ocs palindrome contains two TTACG motifs that could be regarded as variants of the TGACG motif of as-1 (Figure 5B). Furthermore, the two motifs in ocs are arranged in a tail-to-tail inverted repeat. Apparently, the ocs palindrome and the as-1 element of 35S still confer the same tissue-specific expression patterns in vivo even though the sequences are different. These results also suggest that the ocs promoter and the CaMV 35S promoter are controlled by at least one common trans-acting factor.

Singh et al. (1989) found that the ocs palindrome binds a factor present in extracts prepared from Nicotiana plumbaginifolia cell suspension cultures. The binding resulted in the appearance of two bands in gel shift assay. However, a correlation between binding activity and transcription activation was found only with the upper band. The upper band of Singh et al. (1989) may correspond to the single band we observed when the ocs palindrome was incubated with tobacco leaf extracts, although it is possible that the binding activities in leaf extracts and extracts from cell suspension cultures represent different variants of ASF-1.

Our recent analysis of the CaMV 35S promoter revealed that this nominally constitutive promoter is in fact made up of at least two domains, each with a different tissue specificity (Benfey, Ren, and Chua, 1989). Our results...
Figure 4. Factor Binding to the ocs Palindrome.

(A) Binding of a factor from tobacco nuclear extracts. The rbcS promoter sequence between −8 (HindIII) and −166 (BglII) from the rbcS/ocs-wt and rbcS/ocs-mu constructs were eluted and end-labeled with Klenow. The labeled fragments were purified from a gel. ocs wild-type (W) and mutant (M) probes (40,000 cpm) were incubated with (+) or without (−) tobacco nuclear extracts and electrophoresed as described (Katagiri, Lam, and Chua, 1989). Wild-type (W) and mutant (M) sequences used as competitors are indicated. A 400-fold molar excess of competitor over probe sequence was used in each case. The Hex1, as-1, and nos1 sequences (Methods) were prepared for competition experiments as described (Katagiri, Lam, and Chua, 1989). Arrow points to the specific DNA/protein complex.

(B) Binding of TGA1a to the ocs enhancer. Wild-type (W) and mutant (M) ocs probes, as described in (A), were incubated with protein extracts (1 μg) from E. coli expressing the TGA1a protein (+) under the control of a T7 polymerase promoter (F. Katagiri, unpublished data) or with control protein extracts (1 μg) from E. coli not expressing TGA1a (C). Binding of TGA1a was performed under the same conditions with a promoter fragment of rbcS-3A (−166 to −55) containing an as-1 sequence replacing nucleotides −109 to −89 of rbcS-3A (Katagiri, Lam, and Chua, 1989). The free, unreacted labeled fragments (−) were electrophoresed next to the reacted fragments.

Figure 5. Comparison of CaMV 35S as-1 Element and the ocs Palindromic Sequence.

(A) The ocs palindrome (−193 to −178).
(B) The as-1 sequence (−83 to −63).
Arrows show the TGACG motifs in the as-1 sequence and the closely related TTACG motifs in the ocs palindrome.

show that the expression conferred by an ocs DNA regulatory element is confined to the root tip and shoot apex of transgenic plants. Therefore, we suggest that the constitutive expression of the octopine synthase gene in leaves, roots, and stems of transgenic plants (Otten et al., 1981) may also be the result of the combination of several cis elements present in the ocs promoter, each responsible for a different expression profile.

METHODS

Cloning of ocs Enhancer Sequences

The following synthetic DNA fragments were cloned into the HindIII, XhoI sites of the pMON505-derived vector X-46-GUS (P. Benfey, unpublished data). This vector is similar to the vector X-90-GUS (Benfey and Chua, 1989), except that the CaMV 35S promoter sequence is from −46 to +8.

59ocs: ocs promoter sequences between −212 and −154 with the same linker sequences as above.
Upper-case letters denote authentic ocs sequences, whereas lower-case letters represent linker sequences. Asterisks show the bases that differ between the ocs palindrome sequence and its mutant derivative. Numbers indicate borders of the authentic ocs promoter sequences (relative to the transcription start site) present in the constructs.

The following synthetic DNA fragments were cloned into the unique BstXI site at position –55 of the pea rbcS-3A promoter, which contains 166 bp of 5’ upstream sequence with a BgIII linker sequence at the 5’ end. Otherwise, the rbcS construct is the same as the test gene of construct 1 in Kuhlemeier et al. (1988). Insertion of the following ocs sequences at position –55 of the rbcS promoter shifted the rbcS-3A upstream sequences by 21 bp further upstream.

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<th>ocs</th>
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**TGACG-Containing Sequences in the Promoter of rbcS-3A**

Hex1, as-1, and nos1 elements correspond to sequences from the promoters of the wheat histone H3 gene (Mikami et al., 1987), the nopaline synthase gene (An et al., 1986), and the CaMV 35S gene (Lam et al., 1989), respectively. The Hex1, as-1, and nos1 sequences were introduced into the rbcS-3A upstream region, replacing nucleotides –109 to –89 (Katagiri, Lam, and Chua, 1989). Both wild-type (W) and mutant (M) sequences, which were used for competition experiments (Figure 3A), are shown below.

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Hex1 W: 5’ (-190) TCCGCCAGCTGCAACCTGC (-160) 3’
Hex1 M: 5’ (-190) TCCGCCAGCTGCAACCTGC (-160) 3’
as-1 W: 5’ (-83) CTGCTGGATTGCTGACG (-63) 3’
as-1 M: 5’ (-83) CTGCTGGATTGCTGACG (-63) 3’
nos1 W: 5’ (-131) TCAACATGACATACATAC (-111) 3’
nos1 M: 5’ (-131) TCAACATGACATACATAC (-111) 3’
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**RNA Extractions and S1 Nuclease Analysis**

RNA from roots was prepared from plants grown in MS/agar medium containing kanamycin (200 mg/L) and carbenicillin (500 mg/L). RNA from leaves was prepared from fully expanded leaves of plants grown in a greenhouse. RNA extractions and S1 nuclease analysis were performed as described (Fluhry et al., 1986).

**Histochemical Analysis**

Plants for histochemical analysis were grown and tested as detailed by Benfey, Ren, and Chua (1989).

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