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Tissue-Specific Expression of *as-1* in Transgenic Tobacco

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When integrated as a transgene in one or a few copies, the −90 35S promoter of cauliflower mosaic virus confers expression in roots with little or no expression in cotyledons and leaves. The responsible *cis* element, activation sequence-1 (*as-1*), can bind to the nuclear factor ASF-1 as well as to the transcription factor TGA1a. Here, we show that microinjection of 10^4 molecules of TGA1a per cotyledon cell activated transgenes containing *as-1*–linked promoters. Transgenes with promoters linked to the octopine synthase (*ocs*) element, which also binds TGA1a, responded similarly. The acidic, N-terminal segment of TGA1a is important for transcription activation in vivo because a deletion mutant without the first 80 amino acids was inactive. Finally, we show that the −90 35S-β-glucuronidase (*GUS*) fusion gene conferred GUS expression in cotyledon cells when injected at 50,000 copies per cell. Collectively, these results provide support for the hypothesis that the undetectable expression of the *as-1*-linked transgene in cotyledon cells is most likely a result of its inability to compete for a limiting amount of its cognate transcription factor(s), presumably TGA1a or related proteins.

INTRODUCTION

A central problem in research on plant gene regulation is the mechanism of tissue- or cell-type–specific gene expression (Benfey and Chua, 1989, 1990). Investigations of the prototypic 35S promoter of the cauliflower mosaic virus (CaMV) demonstrated that this promoter is, in fact, modular and comprised of several *cis* elements, each of which confers cell-type–specific gene expression in plants (Benfey et al., 1989, 1990a). The apparent constitutive expression of the 35S promoter results from the combinatorial and synergistic interaction of the various *cis* elements (Benfey et al., 1990b). This modular constitution of *cis* elements probably applies to other plant promoters as well, regardless of their mode of regulation.

Initial work on the CaMV 35S promoter showed that it can be divided into two functional domains: A (−90 to +8) and B (−343 to −90) (Benfey et al., 1989). Domain A confers expression mainly in roots, with little or no expression in cotyledon or leaf tissues (Benfey et al., 1989; Salinas et al., 1992). By loss-of-function as well as gain-of-function experiments, the *cis* element responsible for this experiment pattern has been localized to a 21-bp sequence (−83 to −63) designated as activation sequence-1 (*as-1*) (Lam et al., 1989). *as-1* can bind to the nuclear factor ASF-1 and the cloned transcription factor TGA1a (Katagiri et al., 1989). Two other *cis* elements, *octopine* synthase (*ocs*) and *nos-1*, from the promoter regions of Agrobacterium opine synthase genes can also interact with the same factors (Fromm et al., 1989; Lam et al., 1990). Not surprisingly, the expression profile of an *ocs*- or a *nos-1*–linked promoter is similar if not identical to that of *as-1* (Fromm et al., 1989; Lam et al., 1990).

We have used the *as-1* element as a paradigm to investigate the mechanism of cell-type–specific gene expression in plants. RNA gel blot analysis demonstrated that TGA1a is expressed in the leaf at a level at least 10 times lower than that in the root (Katagiri et al., 1989). Accordingly, it was proposed that the low-level expression of *as-1* in leaf cells was a result of a limiting concentration of its cognate factor, presumably TGA1a or related factors, in such cells. Consistent with this notion, it was shown that leaf expression can be obtained by simply attaching four additional copies of *as-1* to the −90 35S promoter (Lam and Chua, 1990). This observation suggests that in leaf cells, *as-1* has to compete with other promoters for its cognate factor.

In this study, we designed experiments to provide further evidence for the competition hypothesis. Using a microinjection technique, we demonstrated that as few as 10^3 molecules per cell of TGA1a were sufficient to activate in vivo promoters linked to its binding sites, *as-1* and *ocs*. Moreover, we provide direct evidence for competition between *as-1*–linked promoters and endogenous promoters in cotyledon cells. Our results indicate that the low-level expression of *as-1* in the leaf was due to limiting concentrations of its cognate transcription factor.

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RESULTS

TGA1a Can Activate Transcription from the −90 35S Promoter

To see if the transcription factor TGA1a could activate the −90 35S promoter in vivo, we purified recombinant TGA1a from *Escherichia coli* and microinjected it into epidermal and subepidermal cells of cotyledons of a transgenic tobacco line carrying the −90 35S–*GUS* transgene. After a 24- to 48-hr incubation, whole seedlings were subjected to histochemical analysis for β-glucuronidase (GUS) activity. Without injection, seedlings of this transgenic tobacco line did not show any GUS activity, except at the root tip and the apical meristem regions (Benfey et al., 1989; Salinas et al., 1992). Injection of 10^4 molecules of TGA1a per cell into individual cotyledon cells activated the transgene in a cell-autonomous manner (Table 1 and Figure 1), whereas injection of an equivalent amount of BSA was unable to activate the transgene. This result indicates that the activation was not simply due to the microinjection process but was dependent on TGA1a. Activation of the promoter by TGA1a required an intact *as-1* element because injection of as much as 1×10^5 molecules of TGA1a per cell activated neither the −72 35S nor the −46 35S promoter (Table 1 and Figure 1). The former lacks the upstream half of the *as-1* element, whereas the latter is completely devoid of the element. The cell-autonomous activation was seen in all cell types of cotyledon and cortical cells and parenchymal cells of the hypocotyl and root (Figure 1).

Taking advantage of the microinjection system, we introduced defined amounts of TGA1a into individual cells. Although 10^4 molecules per cell of TGA1a was able to produce the maximum level of activation efficiency (67.8%, Table 1), as low as 10^3 molecules per cell (1.7×10^{-21} M; assuming a cytoplasmic volume of ∼150 pL) was sufficient to activate the −90 35S promoter in 10% of the injected cells. This observation of cell-autonomous activation of the *as-1*–linked promoter by TGA1a supports the notion that TGA1a or related proteins are limiting for −90 35S promoter transcription in cotyledon cells. Moreover, the use of the microinjection technique enabled us to determine quantitatively the number of TGA1a molecules required to activate this promoter in such cells.

Table 1. Activation of the −90 35S Promoter by TGA1a Requires an Intact *as-1* Element

Promoter of Transgene	Injected Material[a]	No. of Injections	No. of Activations	Efficiency (%)
−90 35S	TGA1a,	648	439	67.8
	BSA	2,205	0	0
−72 35S	TGA1a,	603	0	0
	BSA	180	0	0
−46 35S	TGA1a	148	0	0
No Transgene	TGA1a,	295	0	0
	BSA	180	0	0

[a] TGA1a and BSA were injected at a concentration of ∼10^4 molecules per cell.

Table 2. Activation of a 59-bp *ocs* Upstream Sequence and the *ocs* Element by TGA1a

Transgene	Injected Material[a]	No. of Injections	No. of Activations	Efficiency (%)
ocs 59wt	TGA1a,	369	182	46
	BSA	801	0	0
ocs 21wt	TGA1a,	261	82	31.4
	BSA	402	0	0
ocs 21mu	TGA1a,	495	0	0
	BSA	391	0	0

[a] TGA1a and BSA were injected at a concentration of 10^4 molecules per cell.

TGA1a also Activates a Promoter Linked to the *ocs* Element

The 16-bp ocs element is located in the −193 to −178 region of the *ocs* gene from the Agrobacterium T-DNA region (Ellis et al., 1987). Several lines of evidence suggest that *as-1* and *ocs* are functionally equivalent in tobacco. Sharing extensive DNA homology, these two elements both bind to the nuclear factor ASF-1 as well as to the recombinant TGA1a (Bouchez et al., 1989; Fromm et al., 1989). Moreover, transgenic tobacco plants carrying the *ocs* element linked to a *GUS* reporter gene show an expression pattern very similar to that conferred by *as-1* (Fromm et al., 1989).

We tested whether upstream sequences of the ocs promoter can also respond to TGA1a in a similar way as the −90 35S promoter. Three transgenic tobacco lines carrying transgenes with different promoters but the same *GUS* reporter gene were used in the experiments. The promoters are as follows: (1) *ocs* 59wt is comprised of the −212 to −154 region of the *ocs* promoter, including the *ocs* element, linked to the minimal 35S promoter (−46 to +8); (2) *ocs* 21wt consists of the −193 to −173 region (containing the *ocs* element plus five additional base pairs) of the *ocs* promoter linked to the minimal 35S promoter; and (3) *ocs* 21mu is the same as *ocs* 21wt, except that four mutations were introduced into the *ocs* element, and they block the DNA binding activity of the element, rendering it inactive in vivo (Fromm et al., 1989). All transgenic tobacco lines carrying any of these constructs did not show any detectable GUS activity in cotyledon cells. After 10^4 molecules of TGA1a per cell was injected, the *ocs* 59wt and *ocs* 21wt showed activation of the promoters (Table 2 and Figure 1). By contrast, the *ocs* 21mu plant did not show any activity, even when TGA1a was injected at a concentration of 1×10^5 molecules per cell

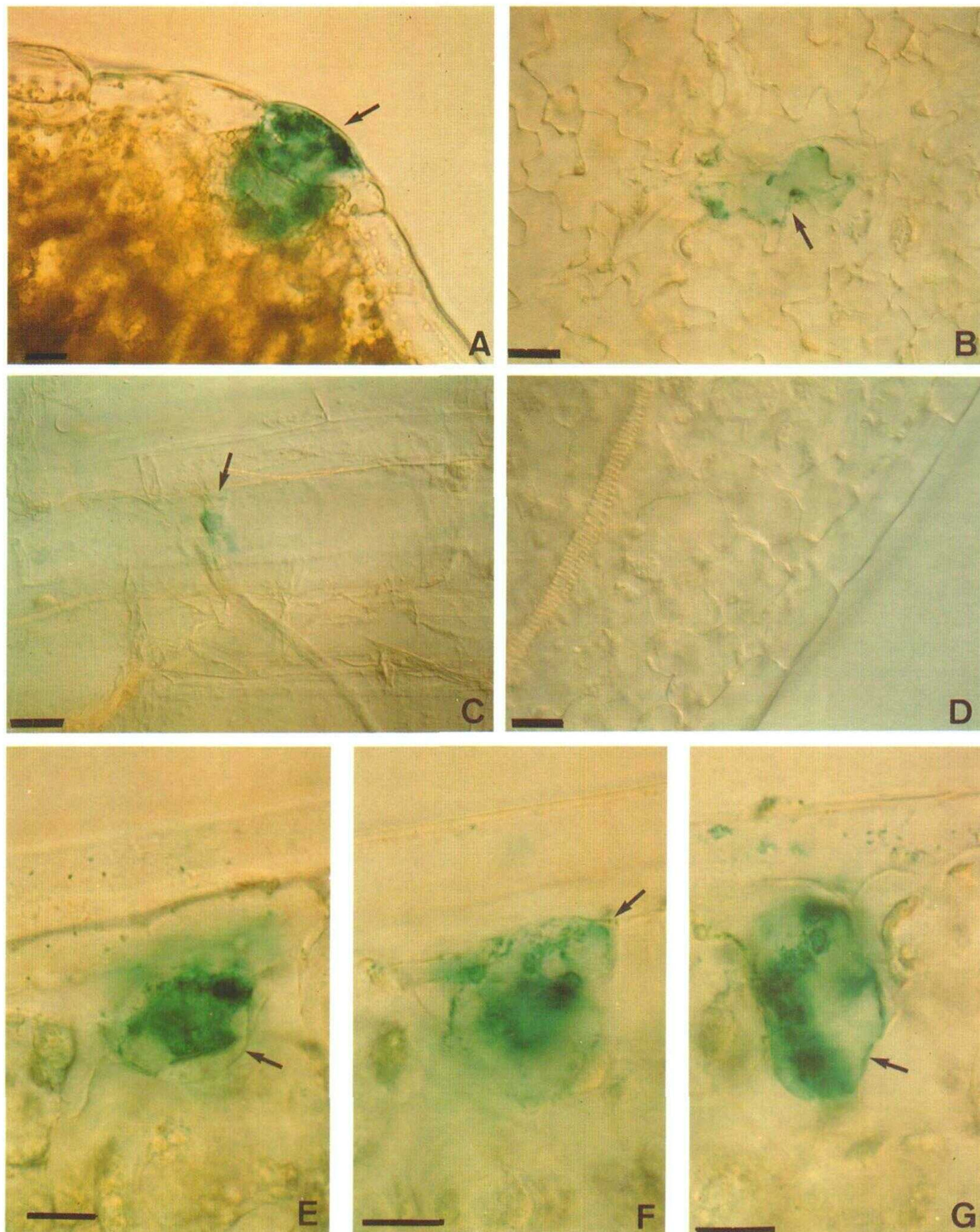

Figure 1. Histochemical Analysis of GUS Activity in Tobacco Cotyledons after Microinjection.

Recombinant TGA1a was injected into cotyledon cells of transgenic tobacco seedlings carrying different promoter constructs linked to the *GUS* coding sequence in (**A**) to (**E**). The concentration of TGA1a was ~10^4 molecules per cell.

(**A**) −90 35S promoter; cotyledon epidermal cell.

(**B**) −90 35S promoter; cotyledon epidermal cell (viewed from the top).

(**C**) −90 35S promoter; root cortical cell.

(**D**) −46 35S promoter; cotyledon subepidermal cell.

(**E**) *ocs* 59wt promoter (Fromm et al., 1989); cotyledon subepidermal cell.

(**F**) RNA encoding TGA1a was injected into a cotyledon subepidermal cell of a transgenic tobacco line carrying the −90 35S–*GUS* transgene. The concentration was ~0.05 pg per cell.

(**G**) Molecules of a plasmid (5 × 10^4) containing the −90 35S–*GUS* fusion gene were injected into a cotyledon subepidermal cell of a wild-type tobacco seedling.

Arrows indicate the injected cell. Bars = 25 μm.

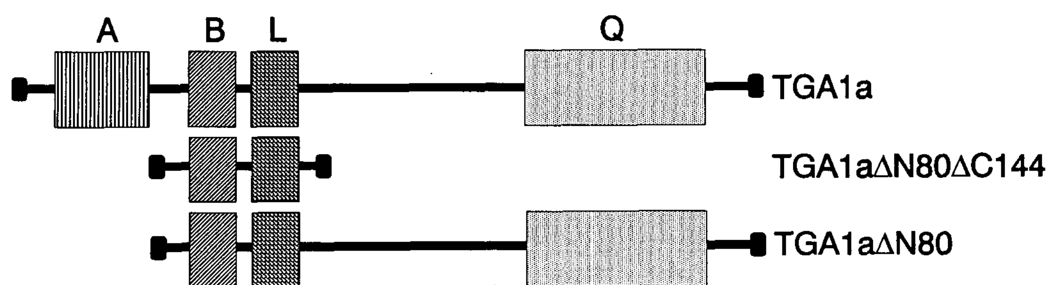

Figure 2. Schematic Representations of TGA1a and Two Deletion Derivatives.

Full-length TGA1a contains 373 amino acid residues. ΔN80 has an N-terminal deletion of 80 amino acid residues, which removes the acidic region (A) but leaves the basic leucine zipper domain and the glutamine-rich region (Q) intact. The deletion of 229 amino acid residues from the C-terminal region of ΔN80 gives ΔN80ΔC144, which still contains the basic leucine zipper domain. B, basic domain; L, leucine zipper.

(Table 2; data not shown). Injection of BSA as a negative control did not cause activation of any of the promoters. Taken together, these results indicate that the promoters linked to *ocs* can be specifically activated by TGA1a in vivo. The efficiency of *ocs* activation appeared to be a little lower than that of *as-1*. The same concentration of TGA1a (10^4 molecules per cell) conferred 67.8% (maximum efficiency) GUS-positive cells with the −90 35S plant and only 46% with the *ocs* 59wt plant (cf. Tables 1 and 2). More rigorous experiments, however, are needed to firmly establish this quantitative difference.

The N-Terminal Region of TGA1a Is Essential for in Vivo Transactivation

We used the microinjection system to investigate the activity of two deletion mutants of TGA1a (Figure 2). Mutant ΔN80 contains an N-terminal deletion of 80 amino acid residues; this region is rich in acidic residues. Removal of 229 amino acid residues from the C terminus of ΔN80 gave ΔN80ΔC144; the deleted segment contains the dimer stabilization region and a glutamine-rich region (Katagiri et al., 1992). Both mutants retain the entire basic leucine zipper domain, which has a nuclear localization signal (van der Krol and Chua, 1991) and a DNA binding function (Katagiri et al., 1992). The DNA binding affinity of ΔN80ΔC144 is at least four times lower than that of the full-length TGA1a, whereas ΔN80 has a binding affinity similar to TGA1a (Katagiri et al., 1992). Table 3 shows that microinjection of 10^4 molecules per cell of these deletion mutants of TGA1a caused activation of neither −90 35S nor *ocs* 21wt. These mutants were inactive even at a concentration of 1×10^5 molecules per cell.

We conclude that the N-terminal segment (amino acids 1 to 80) of TGA1a contains a region essential for in vivo activation of *as-1* and *ocs*. Because this N-terminal region is rich in acidic amino acids, it may function as an activation domain.

Table 3. Activation of −90 35S Promoter and *ocs* Sequences by TGA1a and Its Derivatives

Promoter of Transgene	Injected Material[a]	No. of Injections	No. of Activations	Efficiency (%)
−90 35S	TGA1a,	648	439	67.8
	ΔN80,	621	0	0
	ΔN80ΔC144	612	0	0
−72 35S	TGA1a,	603	0	0
	ΔN80,	593	0	0
	ΔN80ΔC144	609	0	0
ocs 59wt	TGA1a,	396	182	46
	ΔN80	243	0	0
ocs 21wt	TGA1a,	261	82	31.4
	ΔN80	288	0	0
ocs 21mu	TGA1a,	495	0	0
	ΔN80	288	0	0

[a] TGA1a was injected at a concentration of 1×10^4 molecules per cell, whereas the mutant derivatives were injected up to a concentration of 1×10^5 molecules per cell.

Expression of a High Copy Number of the −90 35S-*GUS* Chimeric Gene

Previous work has shown that when integrated into the tobacco nuclear genome in one or a few copies, the promoters −90 35S, *ocs* 59wt, and *ocs* 21wt did not show any detectable activity in cotyledon cells (Benfey et al., 1989; Fromm et al., 1989; Salinas et al., 1992). Here, we demonstrated that the expression of these promoters could be activated by microinjection of TGA1a. This observation is consistent with the notion that a transcription factor(s) responsible for the activity of these related promoters is limiting in cotyledon cells; for example, the concentration of the free factor(s) is far below the dissociation constant between the factor(s) and the *cis* element(s). If this hypothesis were correct, it should be possible to obtain expression of the −90 35S–*GUS* fusion gene in cotyledon cells if the latter could be introduced in multiple copies to bind the transcription factor(s) by mass action or to compete with related *cis* elements in the genome for the same factor(s).

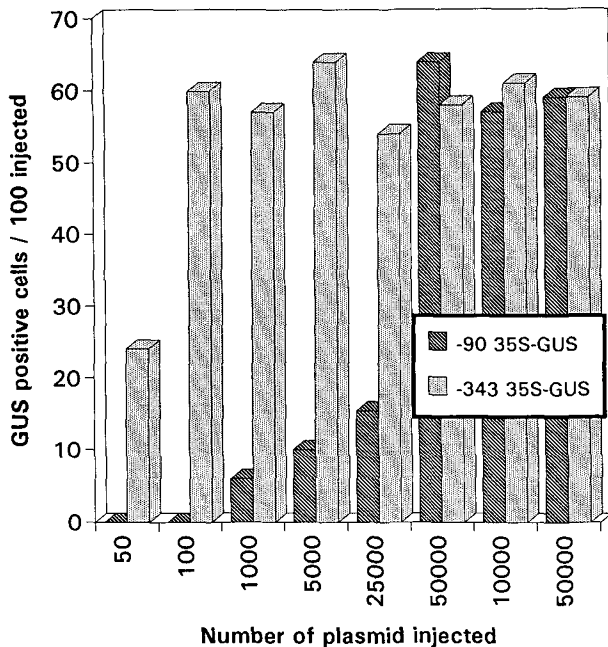

Figure 3. Expression of −90 35S and −343 35S Promoters in Tobacco Cotyledon Cells.

Plasmids containing either the −90 35S or the −343 35S promoter fused to the *GUS* coding sequence were described previously (Benfey et al., 1989). Different amounts of either plasmid were injected into cotyledon cells of wild-type tobacco seedlings. After incubation for 24 to 48 hr, the cotyledons were stained for GUS activity.

To test the above hypothesis, we injected an increasing number of plasmids containing −90 35S–*GUS* into cotyledon cells. A similar plasmid containing the full-length 35S promoter (−343 to +8) fused to the same *GUS* coding sequence (−343 35S–*GUS*) was used as a control. Figure 3 shows that 10^2 molecules of the −343 35S–*GUS* construct was enough to obtain ~60% GUS-positive cells. The percentage of GUS-positive cells did not change significantly, even if the number of injected molecules was increased by 5000 times. This result suggests that the efficiency of ~60% is the maximum efficiency we can statistically obtain under these experimental conditions. In agreement with previous transgenic plant results (Benfey et al., 1989; Fromm et al., 1989), injection of a low copy number (50 to 100 molecules per cell) of the −90 35S–GUS plasmid into cotyledon cells did not produce any detectable activity. GUS-positive cells, however, were seen at a high copy number of the plasmid, and the maximum efficiency of ~60% was obtained with 5 × 10^4 molecules per cell.

Although our results are consistent with the competition hypothesis, we cannot rule out the possibility that a single molecule of the −90 35S promoter has a very weak activity, and the activity we obtained with more than 10^3 molecules of −90 35S per cell was simply the result of an additive effect of gene dosage. To investigate this point further, we injected 5 × 10^4 molecules of the −90 35S–GUS plasmid together with an increasing copy number of the *as-1 cis* element that binds the nuclear factor ASF-1 and the recombinant factor TGA1a. Whereas low concentrations of the *as-1* element had no significant effect, higher concentrations of the competitor reduced the number of GUS-positive cells, and no activity was obtained when the ratio of *as-1*/−90 35S was 10 (Table 4). As a control, we also used as competitor a mutant version of *as-1* (*as-1 mu*), which is severely reduced in its ability to bind ASF-1 and TGA1a (Katagiri et al., 1989). Table 4 shows that 5 × 10^4 molecules of *as-1 mu* had no significant effect on the efficiency of −90 35S–*GUS* expression, whereas the same concentration of *as-1* reduced the number of GUS-positive cells by ~50%. Molecules of *as-1 mu* (1 × 10^5) appeared to slightly decrease the number of GUS-expressing cells. Collectively, these results support the hypothesis that expression of multiple copies of the −90 35S promoter in cotyledon cells was due to the ability of *as-1* in this promoter to compete for transcription factor(s).

DISCUSSION

Transcription Factor for *as-1* Is Limiting in Cotyledon Cells

When integrated into the nuclear genome in one or a few copies, *as-1*– and *ocs*-linked promoters are known to have no detectable activity in tobacco cotyledon cells (Benfey et al., 1989; Fromm et al., 1989; Salinas et al., 1992). In this study, two lines of evidence were found to support the notion that the undetectable expression of *as-1* and *ocs* is most likely a result of a limiting amount of their cognate transcription

Table 4. Expression of the −90 35S Promoter in Tobacco Cotyledon Cells Is Inhibited by the *as-1* Element

Promoter of Injected Plasmid/ Competitor	Copy No. of Injected Combinations	No. of Injections	No. of Activations	Efficiency (%)
−90 35S/*as-1*	50,000/100	80	33	41
	50,000/1,000	85	34	40
	50,000/5,000	85	29	34
	50,000/10,000	125	28	22
	50,000/50,000	125	23	18
	50,000/100,000	125	15	12
	50,000/500,000	160	1	0.6
	25,000/500,000	160	0	0
−90 35S/*as-1 mu*	50,000/100	72	29	40.2
	50,000/10,000	44	20	45.4
	50,000/50,000	81	33	40.7
	50,000/100,000	71	27	37.5
	50,000/500,000	48	21	43.7
	25,000/500,000	51	23	45.0

factor(s), presumably TGA1a or related proteins. First, when injected into cotyledon cells of transgenic plants carrying the appropriate construct, TGA1a activated *as-1*– and *ocs*-linked promoters. Second, −90 35S promoter transcription can be obtained in wild-type tobacco cotyledon cells when introduced at a high copy number. This activity is sensitive to competition by a 21-bp sequence containing the *as-1* element but not the *as-1* mutant, which is severely reduced in its ability to bind ASF-1 or TGA1a. The second observation strongly suggests that the *as-1*–specific factor was not completely absent from cotyledon cells, but rather was present only in limiting amounts. These situations in cotyledons were paralleled by previous observations in leaves, which express neither −90 35S nor *ocs*-linked promoters. It has been shown that the TGA1a mRNA level is 10-fold lower in leaves as compared to roots, where −90 35S and *ocs*-linked promoters are active (Benfey et al., 1989; Fromm et al., 1989; Katagiri et al., 1989). This inactivity of −90 35S in leaves can be overcome by placing four additional copies of *as-1* upstream of this promoter, which presumably increases the probability, additively or synergistically, to interact with the limiting factor (Lam and Chua, 1990).

What could be the role of such a factor(s) that is present in limiting concentrations insufficient to activate *as-1*– or *ocs*-linked promoters? One possibility is that there are endogenous promoters that contain several repeats of the cognate binding sites, such as the artificial promoter with five copies of *as-1* (Lam and Chua, 1990). A second possibility is that endogenous *cis* elements may have a higher binding affinity for the factor. In this connection, it has been shown that TGA1a has a higher binding affinity for a perfect palindromic sequence TGACGTCA as compared to *as-1* (S.-F. Qin and N.-H. Chua, unpublished observations). Another possibility is that the factor for *as-1* may synergize with other *cis* elements in the leaf and cotyledon. Several *cis* elements are reported to show synergistic interaction with *as-1* in leaves (Lam and Chua, 1990), and therefore, the low factor level may still be high enough to be involved in such synergism.

Does TGA1a Directly Activate Transcription in Vivo?

Both TGA1a and *as-1* are necessary for the observed transcription activation. In contrast to in vitro analyses (Katagiri et al., 1990), we cannot be certain whether TGA1a interacts with the *as-1*–linked promoter directly or indirectly in vivo. There are two formal possibilities for indirect interaction: (1) TGA1a displaces an unknown transcription factor X from its binding sites on other promoters, and the released factor X is responsible for the activation of the *as-1*–linked promoter; (2) TGA1a somehow stimulates the expression of another transcription factor, which in turn acts on the *as-1*–linked promoter. The first possibility is unlikely because the deletion mutant ΔN80, which has as high a DNA binding affinity as TGA1a, did not activate the promoter. To test the second possibility, it is necessary to investigate the effects of TGA1a in the absence of protein synthesis. Ordinary transient expression assays require de novo synthesis of transcription factors from effector constructs. By contrast, microinjection of transcription factors as proteins will allow us to measure the effects of the factors in the absence of protein synthesis if expression of the reporter gene is monitored by in situ hybridization of mRNA. Although this second possibility cannot be excluded at the moment, taken together with the in vitro results (Katagiri et al., 1990), it is likely that TGA1a directly activates transcription from the *as-1*–linked promoter in vivo.

Possible Applications of the Microinjection Technique in Gene Expression Studies

In vivo transient expression systems have been widely used to investigate plant transcription factor activities. In such a system, usually an effector and a reporter DNA construct are simultaneously introduced by either electroporation (Lohmer et al., 1991) or microprojectile bombardment (Goff et al., 1990, 1991) into cells in which the factor is not expressed. Although convenient, such techniques are limited to the delivery of DNA only. This limitation, however, can be circumvented by microinjection, which can be used to introduce almost any molecule into specific target cells. We have introduced DNA and a transcription activator protein, as well as RNA encoding the transcription activator (data not shown). This characteristic of the microinjection technique suggests various possible applications. For example, if the activity of a transcription factor is sensitive to modification (e.g., phosphorylation), the factor could first be modified in vitro and then introduced into cells by microinjection to test the effect of modification on its activity. Microinjection can also deliver molecules not efficiently taken up by cells that can potentially affect various biological processes, such as signal transduction (Neuhaus et al., 1993).

Another advantage of microinjection is that single cells can be targeted for analysis. Thus, the assay could be very sensitive, and in addition, it allows us to determine whether the response is cell autonomous (Neuhaus et al., 1993). In the case of the TGA1a/*as-1* combination, the assays were performed with 10^3 to 10^4 molecules per cell of TGA1a. By contrast, an in vitro transcription assay typically requires 10^{11} to 10^{12} molecules (in the range of 10 to 100 ng) for a single reaction mix of 25 μL (Katagiri et al., 1990). This great sensitivity of the microinjection assay would be useful as a transactivation assay for purification of a transcription factor from plant materials.

Under our experimental conditions, the histochemical staining for GUS activity did not allow quantitation of promoter activity. We used the lowest amount of TGA1a protein required for GUS expression in the maximum number of injected cells and/or the percentage of GUS-positive cells as semiquantitative estimates. This problem can be resolved by using the luciferase gene as a reporter gene. The expression of luciferase in a single cell can be measured quantitatively with a microscope linked to a photon counting system (Millar et al., 1992). This technical refinement would allow quantitative measurement of promoter activity in a single cell.

METHODS

Constructs

Four 35S promoter constructs with different 5' sequences were fused to the β-glucuronidase (*GUS*) coding sequence and are as follows: promoter 1, (−46 to +8) plus *GUS* (Benfey et al., 1990a); promoter 2, (−72 to +8) plus *GUS* (Benfey and Chua, 1989); promoter 3, (−90 to +8) plus *GUS* (Benfey et al., 1989); promoter 4, (−343 to +8) plus *GUS* (Benfey and Chua, 1989).

Recombinant Proteins

All injected recombinant proteins, TGA1a, and its derivatives were produced in pET3a (Rosenberg et al., 1987) and isolated according to Katagiri et al. (1990).

Plant Material

Wild-type seedlings of *Nicotiana tabacum* cv SR1 were used for competition experiments. Transgenic tobacco lines carrying the −90 35S-*GUS* transgene were described previously (Benfey et al., 1989), and lines carrying the octopine synthase (*ocs*) 59wt-*GUS*, *ocs* 21wt-*GUS*, and *ocs* 21mu-*GUS* transgenes were obtained as described in Fromm et al. (1989). Transgenic seeds were sterilized with commercial bleach and germinated on Murashige and Skoog (1962) medium (Sigma) containing 3% sucrose, 0.7% agar, and 100 μg/mL kanamycin (Schnorf et al., 1991) under a 16-hr-light/8-hr-dark cycle at 24°C. Seedlings of the appropriate size were used for the experiments.

Histochemical Analysis

Histochemical staining for GUS activity was performed as described previously (Jefferson et al., 1987; Benfey et al., 1989). Staining was allowed to proceed for 12 hr followed by fixation (Benfey et al., 1989). Plant material was mounted on microscopic slides and photographs were taken with an inverted microscope (model IM405; Carl Zeiss AG Schweiz, Zurich, Switzerland) using either bright light illumination or differential interference contrast.

Preparation of Injecting Solution and Microinjection Technique

The material to be injected was dissolved at different concentrations (as given in Results) in an injection buffer containing 10 mM Tris-HCl and 0.1 μM EDTA, pH 7.5. The injection solution was passed through a 0.2-μm disposable filter to sterilize the solution and to remove particles. Injections were carried out under sterile conditions to avoid any contamination. For microinjection, the micromanipulators of the Embryo Splitter from Research Instruments (Basel, Switzerland) were used to facilitate the movements of the injection capillary, which was fixed by a capillary holder on one of the micromanipulators. The injection was done by pressure using the Eppendorf pressure device. Injection volumes were fixed by adjusting the time and the injection pressure on the Eppendorf apparatus (Schnorf et al., 1991). Microinjection was controlled by using a high-magnification (up to 160×) stereomicroscope (model SV11; Carl Zeiss). The seedlings to be injected were placed in a Petri dish containing filter papers wetted with sterile water. The seedlings were kept intact throughout microinjection, and only one of the two cotyledons was injected; the second cotyledon served as a noninjected control. After injection, the seedlings were incubated for 24 or 48 hr in the same Petri dish wrapped with Parafilm (M; American National Can TM, Greenwich, CT).

The system was optimized and controlled by injecting lucifer yellow (Sigma) into cells of seedling cotyledons. The same set-up was used for microinjection into other cell types of the seedlings.

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