Differential Accumulation of a Transcript Driven by the CaMV 35S Promoter in Transgenic Tobacco

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ABSTRACT

A transcriptional fusion composed of the CaMV 35S promoter, a 19 kD zein cDNA, and the 3' flanking regions from the 0' to 1' Tρ T-DNA genes was introduced into tobacco (Nicotiana tabacum) by Agrobacterium-mediated transformation. The accumulation of RNA generated from this transcriptional fusion varied both temporally and spatially in all tissues examined in greenhouse-grown tobacco plants, suggesting that the CaMV 35S promoter is not constitutive. Younger, actively dividing leaf, stem, root, and flower tissues contained higher steady state levels of zein RNA than did older, more quiescent tissues. Zein RNA levels greatly decreased during seed development and were undetectable in the mature seed. In addition, the two RNA termination or processing signals present in our construct were differentially utilized during seed development.

The cauliflower mosaic virus 35S promoter exhibits a high level of transcriptional activity in a variety of plant tissues and is one of the most widely studied and utilized promoters in transgenic plants. Several publications have described elements, including a putative upstream activating sequence (UAS), affecting the transcriptional level of this promoter (21–23). Because 35S promoter activity has been described as “constitutive” (i.e., transcription from the 35S promoter is assumed to be maximal and the same in all cell types [21]), researchers have frequently used this promoter as a control in studies of the regulation of other promoters (3) as well as in the construction of plant expression vectors (2) and chimeric selectable marker genes (15). Two recent studies suggest, however, that the expression of genes fused to the 35S promoter may not be constitutive. Nagata et al. (20) have shown that the activity of the 35S promoter is S phase specific in tobacco protoplasts, and Jefferson et al. (13) have shown that the expression of β-glucuronidase (GUS) activity directed by a 35S-GUS transcriptional fusion is limited to the vascular cambium in stems of transgenic plants. In the present study, we examined the accumulation of a transcript driven by the 35S promoter in various tissues and at various developmental stages in transgenic tobacco plants. The accumulation of this transcript varies both temporally and spatially in all tissues examined, suggesting that 35S promoter activity is not constitutive.

Received for publication February 2, 1989

and in revised form April 7, 1989

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from Bethesda Research Laboratories and used according to manufacturer's specifications. Klenow fragment of DNA polymerase I and T4 DNA ligase were obtained from Pharmacia. [α-32P]dCTP was purchased from Amersham, and DNA fragments were labeled using an Amersham random-primer labeling kit.

Plasmid Constructions and Bacterial Growth Conditions

We constructed the 35S-zein chimeric gene p35SZ as shown in Figure 1. We first isolated an 831 bp HinII fragment containing a 19 kD zein coding sequence from the cDNA plasmid pZ19c1 (Fig. 1) (18), treated it with Klenow fragment of DNA polymerase I to produce blunt ends, and inserted it into the SmaI site of pUC18 to produce pUC19c1 (Fig. 1, step 1). We then digested the plasmid pUC19c1 with BamHI and XbaI and inserted a 450 bp2 BamHI-XbaI fragment containing the CaMV 35S promoter and 5' untranslated region from the plasmid pCANZAN95 (a kind gift of Dr. M. Fromm) (Fig. 1, step 2) to produce pUC3SSZ. We digested the binary plant transformation vector pGA492 (1) with HpaI and inserted a 700 bp HinII fragment containing a bidirectional polyadenylation sequence from the region between the Agrobacterium tumefaciens Tρ T-DNA genes 0' and 1' (14) (Fig. 1, step 3), producing plasmid pGApA. Finally, we digested pGApA with HindIII and SstI and inserted the HindIII-SstI fragment containing both the 35S promoter and 19 kD zein coding sequence from pUC35SZ (Fig. 1, step 4), producing the plasmid p35SZ.

We have described the construction of the phaseolin-zein chimeric gene pSpA previously (29). pSpA contains the same 19 kD zein gene as p35SZ but is flanked by the promoter and polyadenylation sequences from a β-phaseolin gene (26). Both plasmids contain a nos-nptII fusion that confers kanamycin resistance upon plants. We mobilized the recombinant plasmids into A. tumefaciens strain LBA4404 (11) by triparental mating (7).

We introduced the plasmid pUC19c1 into Escherichia coli strain JM103. All other recombinant DNA manipulations were performed in E. coli strain HB101. E. coli were grown at 37°C on Luria broth medium (19). When required, ampi...
cilin was added to a concentration of 50 μg/mL, and kanamycin to a concentration of 100 μg/mL. Matings were performed at 30°C on yeast-extract-peptone medium (16). *A. tumefaciens* strains were grown at 30°C on AB sucrose minimal medium (16) with kanamycin added to a concentration of 100 μg/mL when required.

**Plant Transformation, Regeneration, and Growth**

We infected leaf discs from *Nicotiana tabacum* line W38 with *Agrobacterium* strains harboring the plasmid p35SZ or pSpA. Leaf disc infections, the selection of kanamycin-resistant calli, and the regeneration of transgenic plants were carried out as previously described (29).

We transferred transformed plants to potting soil and grew them under greenhouse conditions. Flowers were manually self-pollinated using a sterile paintbrush, and seed was collected at 5, 10, 15, 20, and 25 dpp. Duplicates of these same transgenic tobacco plants containing p35SZ were also grown aseptically in BGS propagation medium. BGS medium contains MS salts (4.3 g/L), sucrose (30 g/L), B1 vitamins (0.4 mg/L), inositol (0.1 g/L), folic acid (0.001 μg/L), IAA (0.1 μg/L), and kinetin (0.03 μg/L). All plant material used for these analyses was harvested at the same time and stored at -70°C to eliminate possible variations due to circadian rhythms or other environmental signals.

**Analysis of DNA and RNA**

We extracted total DNA from leaves of transformed and nontransformed tobacco plants by the method of Dellaporta *et al.* (6), and used the procedure of Southern (27) to detect the presence of the chimeric zein genes. Total DNA (15 μg) was digested with *EcoRI* and *HindIII*, separated by electrophoresis through a 1.2% agarose gel and transferred onto nitrocellulose. The blots were hybridized with an 850 bp *EcoRI-HindIII* fragment containing the zein coding sequence from clone pUC19c1 (Fig. 1). We performed the hybridizations and autoradiography as described previously (10).

We analyzed the expression of the chimeric genes in transformed plants by Northern blot analysis (17). Total RNA was extracted from leaves, stems, roots, petals, and seeds of tobacco as previously described (29). Poly(A)* RNA was isolated using poly(U) Sephadex affinity chromatography (Bethesda Research Laboratories). Total RNA (10 μg) or poly(A)* RNA (5 μg) was separated by electrophoresis through 1.2% formaldehyde-agarose gels and transferred to nitrocellulose. The blots were hybridized with either an 850 bp *EcoRI-HindIII* fragment containing the zein coding sequence of pUC19c1, or a 700 bp fragment containing the 0' to 1' polyadenylation sequence from pGA492 (p35SZ) (1) and used the plasmids as described (29).

We cloned the chimeric genes into the binary plant transformation vectors pGA492 (p35SZ) (1) and pBin9 (pSpA) (4), mobilized the plasmids into *A. tumefaciens* strain LBA4404 by triparental mating, and used the resulting transconjugants to infect tobacco leaf discs (12). We verified the transformation of regenerated kanamycin-resistant plants by Southern blot analysis, and selected several independent transformants containing from one to five copies of each gene for further analysis. Plant p35SZ-2, which is shown as our 'representative' plant, contains a single copy of our chimeric 35S-zein gene.

**Analysis of Zein RNA Accumulation in Tobacco Plants Maintained in Culture**

We initially assayed the accumulation of zein RNA directed by the 35S promoter in three independent transgenic plants maintained in BGS medium. Northern blot analysis of total RNA from all of these plants showed that the accumulation of zein RNA was approximately the same in upper and middle leaves, as well as in the upper and lower portions of the roots. The results from a representative plant are shown in Figure 2. These findings agree with those of An *et al.* (3) and Odell *et al.* (21) and suggest that in tobacco plants maintained on propagation medium supplemented with the phytohormones IAA and kinetin, 35S promoter activity is indeed constitutive.

**Differential Zein RNA Accumulation in the Vegetative Tissues of Greenhouse-Grown Plants**

When we analyzed the accumulation of the zein transcript in tissues from these same plants grown under greenhouse conditions in the absence of exogenous phytohormones, we noted marked differences. We subjected total (Figure 3) or poly(A)* RNA (not shown) from various tissues of three independently transformed, greenhouse-grown p35SZ to-

**RESULTS**

**Construction of Chimeric Genes Containing the 19 kD Zein Coding Sequence**

The two chimeric genes used in this study are transcriptional fusions between a 19 kD zein coding region and either a CaMV 35S promoter or the promoter from a β-phaseolin gene. The zein coding sequence used in these constructions derives from a cDNA clone (18) and does not contain an efficiently utilized complete polyadenylation consensus sequence. We have observed that tissues transformed with chimeric genes containing this 19 kD zein coding sequence without an additional polyadenylation sequence accumulate very low levels of the zein transcript. However, plants transformed with chimeric genes that contain an additional, complete polyadenylation sequence accumulate much higher levels of the zein transcript (our unpublished results). Therefore linked the cDNA to polyadenylation consensus sequences from one of two sources. The plasmid p35SZ incorporates a CaMV 35S promoter and a bidirectional polyadenylation sequence from the region between the *Agrobacterium tumefaciens* Tκ T-DNA genes 0' and 1' (14) (Fig. 1). The plasmid pSpA utilizes both the promoter region and polyadenylation consensus sequence from a β-phaseolin gene (26, 29).

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bacco plants at various stages in development to Northern blot analysis. We also analyzed pooled RNA from petals and roots of five independent transformants. The results from a representative plant are shown in Figure 3. Zein RNA comprised a higher proportion of the total and poly(A)+ RNA populations in younger, expanding leaves than in older leaves that have reached their maximum size (Fig. 3A). A gradient of accumulation of zein RNA existed between the two extremes. Zein RNA was also present at a higher level in the total RNA population of younger flower petals than in older petals (Fig. 3B, lanes 1 and 2). In older petals the more rapidly expanding upper portion of the corolla contained a slightly higher level of zein RNA than did the lower portion (Fig. 3B, lanes 3 and 4). Zein RNA was also present at a higher level in the total RNA population of younger (lower) root tissue than in older (upper) root tissue (Fig. 3B, lanes 5 and 6). These trends were seen both in pooled RNA samples and in samples from individual plants.

We detected transcripts of two different sizes in plants transformed with p3SSZ when we hybridized Northern blots of the above tissues with a fragment containing the zein coding sequence (Fig. 3A). The larger, more abundant transcript (about 1250 nt) corresponded in size to a RNA initiating correctly at the 3SS transcriptional start site and terminating at the Tr T-DNA polyadenylation site in the chimeric gene. The smaller message (about 1000 nt) was present at a much lower level. When we hybridized these same blots with a portion of the 3' region of the chimeric gene containing only the Tr T-DNA polyadenylation signal sequence, we detected only the larger transcript (data not shown). This finding suggests that the shorter zein transcript did not extend to the

**Figure 1.** Construction of the 35S-zein chimeric genes. The plasmids and cloning steps (1 through 4) used in the construction of p3SSZ are described in "Materials and Methods." Solid arrows indicate the direction of transcription. A. Alu; B. BamHI; BglII; C. Clal; E. EcoRI; Hc. HindIII; H3. HindIII; Hf. HinfI; Hp. Hpal; K. KpnI; Pv. PvuII; Sm. SmaI; Ss, SstI; X. XbaI.

**Figure 2.** Zein RNA accumulation in tobacco plants maintained in culture. Total RNA (10 μg) from leaves and roots of several plants independently transformed with p3SSZ was separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose. The blot was hybridized with a 1.7 kb EcoRI-HindIII fragment from pSpA (29) containing the zein coding sequence. Results for a representative plant, p3SSZ-2, are shown. Lanes 1 through 6 contain RNA from the shoot apex, leaves 1, 3, and 5 nodes removed from the apex, and upper and lower root sections (root base and tip, respectively). Lanes 7 through 10 contain zein RNA prepared by the in vitro transcription of the plasmid pSP6.cZ19dΔwt with SP6 RNA polymerase (9). These samples correspond to the amount of zein RNA comprising 0.05, 0.1, 0.5, and 1.0% of the poly(A)+ RNA in the loaded samples. The sizes of the RNAs are shown at the right.
Differential Zein RNA Accumulation in Leaf Sections

We next examined the spatial pattern of accumulation of zein transcripts in 8, 23, and 40 cm leaves of transgenic plants harboring the 35S-zein chimeric gene. Leaves from three independent transformants were sectioned as described in Figure 4A. For 8 cm leaves we also examined pooled RNA samples from five independent transformants (data not shown). The results from a representative plant are shown. We analyzed only tissue from the leaf blade to eliminate any possible effect of secondary growth in major leaf veins (8). In 8 cm leaves, a stage of leaf development during which leaves accumulated high levels of zein transcript (Fig. 3) the level of zein RNA was slightly higher in the middle sections of the leaf than at either the tip or base (data not shown). We saw this pattern of differential accumulation in 8 cm leaves for both pooled RNA samples and samples isolated from individual plants. In 23 cm leaves, in which the level of accumulation of the zein transcript was considerably lower than in 8 cm leaves, we detected a much more distinct pattern of differential zein RNA accumulation (Fig. 4B). Cross-sections of the 23 cm leaves showed that the level of zein RNA was again much higher in the middle sections of the leaf than at either the tip or the base. We saw a similar, though less dramatic, pattern of differential zein accumulation in 40 cm leaves (Fig. 4B). Relative levels of zein RNA accumulation are shown in Figure 4C.

Differential Accumulation of 35S Promoter Driven Transcript during Seed Development in Greenhouse-Grown Plants

The differential accumulation of zein RNA in greenhouse-grown plants was most marked in the developing seed. We analyzed total RNA from developing seed of three independent plants transformed with either p35SZ or pSpA at 5, 10, 15, 20, and 25 dpp. Results from a representative plant of each type are shown in Figure 5. In vegetative tissues, we again detected transcripts of two different sizes in the seeds of p35SZ plants when we hybridized Northern blots with a fragment containing the zein coding sequence. The larger, more abundant transcript (about 1250 nt) was present at high levels in developing tobacco seeds at approximately 5 dpp. The level of this RNA species declined rapidly thereafter; the larger zein RNA was not detectable by Northern blot analysis in dry seed (Fig. 5A). As in vegetative tissues, the smaller message (about 1000 nt) was present at a much lower level than the larger transcript, but accumulated only during the later stages of seed maturation. This smaller RNA began to accumulate at approximately 10 dpp and reached a maximum at approximately 20 dpp, at which time it constituted approximately 50 to 75% of the total zein mRNA. When we hybridized these same blots with a portion of the 3' region of the chimeric gene containing only the T<sub>R</sub> T-DNA polyadenylation signal sequence, we detected only the larger transcript (Fig. 5B). This finding suggests that the shorter zein transcript did not extend to the T<sub>R</sub> T-DNA polyadenylation signal sequence. This observation suggests not only that two different RNA termination or processing signals are utilized in these transgenic tobacco plants, but that they are differentially utilized during seed development.

In contrast, zein RNA synthesis directed by phaseolin promoter-zein transcriptional fusions resulted in the accumulation of a single abundant transcript of approximately 1000 nt that did not appear until approximately 10 dpp in the seeds of pSpA transformed tobacco plants (Fig. 5C). In these plants the relative levels of zein RNA continued to increase throughout seed maturation, and high levels of zein RNA remained in the mature seed. The maximum level of zein RNA (about 2–3% of the total messenger RNA) in the seeds of tobacco plants containing phaseolin-zein chimeras was several times higher than the highest levels (about 0.5–1% of the total messenger RNA) of zein RNA observed in any tissue of plants transformed with the 35S-zein chimeric gene.

DISCUSSION

The transcription of chimeric genes directed by the cauliflower mosaic virus 35S promoter has often been thought to be similar in all cell types and during all stages of plant development. In dividing tobacco protoplasts, however, 35S
promoter activity is S phase specific (20). In addition, Jefferson et al. (13) showed that the expression of β-glucuronidase (GUS) activity directed by a 35S-GUS transcriptional fusion was limited to the vascular cambium in stems of transgenic plants. Our results showed that in plants transformed with a 35S-zein transcriptional fusion and grown in BGS propagation medium supplemented with IAA and kinetin, the accumulation of zein RNA did indeed appear to be similar in all vegetative tissues examined (Fig. 2). However, in tissues from greenhouse-grown plants, zein RNA comprised a much higher proportion of both the total and poly(A)+ RNA populations in younger, expanding leaves than in older leaves (Fig. 3A). Because our results were essentially the same for both total and poly(A)+ RNA, these observations were not simply the result of a decrease in the poly(A)+/total RNA ratios as leaves matured. This trend was also seen in developing roots, stems, and petals, where zein RNA comprised a successively smaller portion of the total RNA in these tissues as they matured (Fig. 3B). Analysis of lateral sections of 23 and 40 cm leaves showed that the level of zein RNA was much higher in the middle sections of the leaf than at either the tip or the base (Fig. 4). Because examination of several independent transformants showed identical trends in zein RNA accumulation, these results most likely are not the result of position effects.

We detected the most marked differences in zein RNA accumulation in developing seeds of greenhouse-grown tobacco plants (Fig. 5). A high level of zein RNA was seen at 5 d postpollination. Zein RNA levels declined rapidly thereafter and were below the level of detection by Northern blot analysis in mature seed. The differential accumulation of zein RNA could be caused by the differential stability of this RNA species during seed development. The pattern of zein RNA accumulation directed by the β-phaseolin promoter in transgenic tobacco seed, however, suggests that this is not the case. Transgenic tobacco plants harboring the phaseolin-zein chimeric gene, pSpA, did not begin to accumulate zein RNA until approximately 10 to 12 dpp (Fig. 5C). The concentration of zein transcript increased until approximately 18 to 20 dpp and remained high throughout the period of seed desiccation. This pattern of zein mRNA accumulation paralleled that of β-phaseolin RNA accumulation in the seed of transgenic tobacco plants harboring a β-phaseolin gene (26). These results, therefore, suggest that differential zein mRNA stability is not the cause of the low levels of zein RNA detected in the later stages of seed development in transgenic tobacco plants harboring the 35S-zein chimeric gene.

In all the tissues that we examined for which information on mitotic activity is available, there is a good correlation between higher levels of 35S driven zein RNA accumulation and cell division (5, 24, 25, 28). Together with the observations of Nagata et al. (20) that 35S promoter activity is S phase specific in dividing protoplasts and Jefferson et al. (13)
that the expression of a 35S-GUS transcriptional fusion was limited to the vascular cambium in stem sections, our data suggest that 35S promoter activity might similarly be restricted to actively dividing tissues in whole plants.

If 35S promoter activity does indeed correlate with cell division or DNA replication, we might expect to see an additional peak of zein mRNA accumulation during early embryo formation (about 19–20 dpp), a period that is also characterized by rapid cell division. Because the embryo constitutes only a very small proportion of the developing tobacco seed, we might not expect to be able to distinguish accumulation of zein RNA in the embryo from residual RNA produced in the endosperm unless the RNA produced in embryo tissues is of a significantly different size from the RNA produced in the endosperm. We did in fact note the presence of two different sizes of zein mRNA (about 1250 and 1000 nt) in both the seeds and vegetative tissues of the p35SZ plants. Experiments are in progress to determine whether or not this smaller zein message accumulates preferentially in the embryo during seed development.

One trivial explanation for the observation that seeds in these plants accumulate a 1000 nt zein RNA during the later stages of seed development might be that a copy of the phascolin-zein chimeric gene (pSpA) was introduced into plants containing p35SZ by inadvertent cross-pollination with other plants in the greenhouse that contain pSpA. The pSpA chimeric gene produces a transcript of approximately 1000 nt at approximately the same time in seed development during which our minor transcript begins to accumulate in plants containing p35SZ. This is apparently not the case, because this smaller RNA species is also seen in the leaves, roots, and stems of the same plants. The phaseolin promoter is not active in these tissues (26). In addition, because these plants are primary transformants, genes introduced into the developing seed by cross-pollination would not be present in vegetative tissues. Another possibility might be that the message for an endogenous tobacco protein hybridizes with the 19 kD zein coding region under stringent hybridization conditions. This also apparently is not the case, because total RNA from developing seed at 19 to 20 dpp from untransformed W38 tobacco plants does not hybridize with a zein probe (data not shown).

The larger, more abundant transcript (about 1250 nt) seen in both seeds and vegetative tissues corresponded in size to a RNA initiating correctly at the 35S transcriptional start site and terminating at the T_R T-DNA polyadenylation site in the chimeric gene. The smaller (about 1000 nt), lower-abundance transcript corresponded in size to a RNA initiating at the normal 35S transcriptional start site but terminating near the zein polyadenylation consensus sequence, and not at the T_R T-DNA polyadenylation sequence. A fragment corresponding to the 3' region of the chimeric gene containing only the T_R T-DNA polyadenylation signal sequence hybridized only with the larger transcript (Fig. 5B), further suggesting that the shorter zein transcript did not extend to the T_R T-DNA polyadenylation signal sequence. It thus appeared that two different RNA termination or processing signals were utilized at different stages in development in these transgenic tobacco plants. This is, to our knowledge, the first example of the developmentally regulated differential polyadenylation of a mRNA in plants.

The apparent differential accumulation of RNA directed by the 35S promoter might have several significant implications. For example, experiments leading to the identification of a 35S upstream activating sequence were performed in regenerating protoplasts (22). If 35S promoter activity is dependent on changes in DNA conformation accompanying replication, the significance of this sequence might be quite different from that proposed. In addition, because both crown gall tumors and calli grown on tissue culture medium contain plant growth regulators that stimulate cell division, the use of the 35S promoter as a reference for the analyses of the relative strengths of constitutive promoters in these tissues may be biased. This may be particularly true of promoters such as the nos promoter that appears to exhibit lower transcriptional activity in younger actively dividing tissues (3). Finally, because the accumulation of RNA directed by the 35S promoter appeared to vary not only from tissue to tissue within the same plants but also during different stages of development within the same tissue, this might affect the use of this promoter in expression vector construction.

Figure 5. Differential accumulation of zein RNA during seed development in greenhouse-grown plants. Total RNA was extracted from the seed of several plants independently transformed with p35SZ (A and B) or a phaseolin promoter-zein cDNA chimeric gene, pSpA (C) at various times postpollination, separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose. A representative plant for each construct (p35SZ-2 and pSpA-12) are shown. Lanes 1 through 6 contain total RNA (10 μg) from tobacco seed 5, 10, 15, 20, 25, and 30 d postpollination. Lanes 7 through 9 contain in vitro transcribed zein RNA equivalent to 0.1, 0.5, and 1.0% of the poly(A) "RNA in each lane, respectively. The Northern blots in panels A and C were hybridized with a DNA fragment of p35SZ containing only the zein coding sequence. The blot in panel B was hybridized with a fragment containing only the 3' flanking regions from the 0' to 1' T_R T-DNA genes. The RNA standards in panel B were hybridized separately with an equivalent amount of the labeled zein coding sequence.
ACKNOWLEDGMENTS

The authors wish to thank Dr. Mike Fromm for kindly providing plasmid pCANZAN95 and Dr. Gadi Galili for zein RNA standards. We are also grateful to Drs. Ann Matthesy and Susan Karcher and to Brad Goodner for critical comments and discussion, and Ms. Wilma Foust for help in preparation of the manuscript.

LITERATURE CITED