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A DNA sequence element that confers seed-specific enhancement to a constitutive promoter

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Genes encoding β-conglycinin, a soybean seed storage protein, are expressed only in seeds during mid-to-late stages in embryogeny. It was previously determined that a DNA sequence ~200 nucleotides upstream of the transcriptional start site of the gene encoding the α'-subunit of β-conglycinin is essential for regulated gene expression in transgenic plants. The regulatory effect of this DNA element was tested by inserting the element in different positions and different orientations within a chimeric constitutively expressed reporter gene. The reporter gene was comprised of the 35S promoter from cauliflower mosaic virus (CaMV), a gene encoding chloramphenicol acetyltransferase (CAT) and the polyadenylation signal from the α'-subunit gene. The element had no significant effect on the expression of the CAT gene in roots, stems, or leaves, regardless of the position of its insertion (i.e. 5' or 3' of the gene). However, there was 25- to 40-fold enhancement of CAT gene expression in seeds during mid-to-late stages of embryo development when the element was placed in either orientation within the 35S promoter. There was 2- to 4-fold enhancement of CAT activity when the element was placed 3' of the CAT coding sequence. No enhancement was detected when the element was placed downstream of the 3' non-coding region. This is, to our knowledge, the first identification of a *cis*-acting element that enhances gene expression in a tissue-specific and temporally regulated manner during embryo development in plants.

Key words: β-conglycinin/seed specific enhancer element/35S promoter/transgenic plants

Introduction

A number of different plant genes that exhibit highly regulated expression either in specific cell types, or in certain stages of development, or in response to physiological stimuli are under study (see Kuhlemeier *et al.*, 1987b, for review). Recent technical advances that made possible the introduction of DNA segments into transgenic plants have provided the means to assess the function of *cis*-acting elements that regulate plant gene expression. Timko *et al.* (1985) reported that a 900-bp DNA element from the upstream region of ss3.6, a photoregulated gene, contains the information necessary for maximum gene expression and photoregulation. This element can function independently of orientation but only when placed 5' to the reporter gene. A similar upstream element (−330 to −50) is found in another photoregulated gene, *rbcS*-3A (Fluhr *et al.*, 1986). Recently it was reported that a 58-bp sequence in the *rbcS*-3A promoter between nucleotides −169 and −112 can function as a negative regulatory element (Kuhlemeier *et al.*, 1987a). *cis*-acting elements have been identified in several plant genes, including those that respond to light (pea *cab* gene, Simpson *et al.*, 1986), to UV light (chalcone synthase gene, Kaulen *et al.*, 1986), to heat shock (soybean heat shock gene, Baumann *et al.*, 1987), to anaerobiosis (maize *Adh*-I gene, Ellis, 1987), and those that are expressed during embryo development (soybean storage protein gene, Chen *et al.*, 1986).

The synthesis of soybean seed storage proteins is confined to stages in embryo development of cell enlargement and seed maturation (Meinke *et al.*, 1981; Hill and Breidenbach, 1974). The abundance of these proteins and their mRNAs make this an excellent model system for studies of temporal and tissue-specific gene regulation. β-Conglycinin is a major storage protein of soybeans and contains α', α, and β subunits. Expression of genes that encode these subunits is under strict tissue-specific and temporal control during soybean embryogenesis (Meinke *et al.*, 1981; Walling *et al.*, 1986). When genes encoding the α' and β subunits were transferred into petunia (Beachy *et al.*, 1985; Naito *et al.*, in press; Bray *et al.*, 1987) and tobacco plants (Chen and Beachy, unpublished), each was expressed only in developing seeds. While the α'-gene transcripts begin to accumulate in petunia seeds ~12 days after pollination, transcripts of the β gene were detected ~2 days later than the α' gene. This order of expression of the α' and β genes is similar to that reported for their expression during soybean seed development (Naito *et al.*, in press; Tierney *et al.*, 1987).

To identify regulatory *cis*-acting elements that control these functions in the α' gene, a series of deletion mutations were made in the 5'-flanking sequence of the α' gene and these constructions were introduced into petunia plants (Chen *et al.*, 1986). A low level of expression of the α' gene occurred in developing seeds of transgenic plants that contain the α' gene flanked by 159 nucleotides 5' of the transcriptional start site. A 20-fold increase in expression occurred when an additional 98 nucleotides of upstream sequence were included. The DNA sequence between nucleotides −143 and −257 contains 5 repeats of the sequence $A^{A}_{G}CCCA$, and we have suggested that these elements may play a role(s) in conferring tissue-specific and developmental regulation to the α' gene (Chen *et al.*, 1986).

A 170-bp DNA sequence (−78 to −257) was placed in different positions and orientations into a chimeric gene comprised of the 35S promoter from cauliflower mosaic virus (CaMV) linked to a reporter gene. The 35S promoter is a strong constitutive promoter in transgenic plants (Sanders *et al.*, 1987) and has been widely used in plant transformation experiments (Lawton *et al.*, 1987; Ow *et al.*, 1986;

Powell Abel *et al.*, 1986). We report here the results of experiments showing that the 170-bp upstream DNA element isolated from the α′-gene promoter can enhance expression of the CaMV 35S promoter in a tissue-specific and temporally regulated manner.

Results

Plasmid construction and transformation of tobacco plants

The 35S promoter of CaMV was linked to the coding region of the gene encoding CAT as described in Materials and methods. The polyadenylation signal was provided by the 3′ non-coding region of the α′ gene (pCB6-36) (Figure 1).

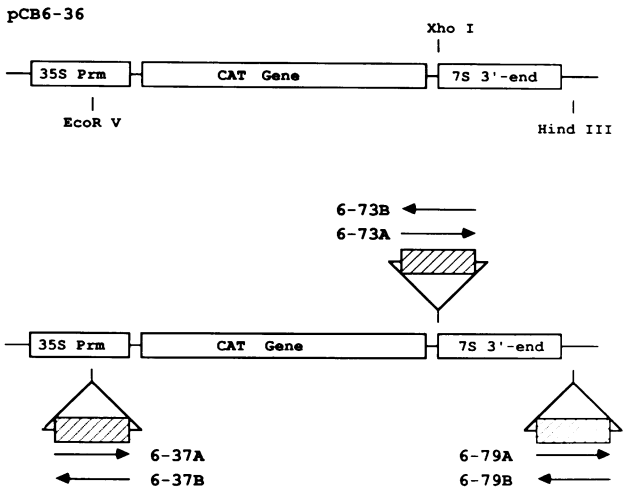

Fig. 1. Construction of the chimeric genes. pMON316 (Sanders *et al.*, 1987), which contains the 35S promoter of cauliflower mosaic virus and the nopaline synthase 3′ end, was used as the intermediate vector. The CAT gene was inserted into the polylinker region. The 3′-non-coding sequence from the α′ gene (*Gmg* 17.1; Doyle *et al.*, 1986) was used as the polyadenylation control region (pCB6-36). The upstream DNA element (−78 to −257) of the α′ gene (Chen *et al.*, 1986) was inserted into the 35S promoter at position −90 (pCB6-37), 3′ to the CAT coding sequence (pCB6-73) or downstream α′ gene 3′ non-coding region (pCB6-79) as described in Materials and methods. The element was inserted such that it was in the same (A) or inverse (B) orientation relative to the direction of transcription.

The chimeric gene was assembled in pMON316 (Sanders *et al.*, 1987), an intermediate vector for plant transformation. The upstream DNA sequence (−78 to −257) isolated from the α′-gene promoter was subcloned and inserted into the 35S promoter at position −90 either in the proper (pCB6-37A) or reverse (pCB6-37B) orientation relative to the direction of transcription. The fragment was also inserted 3′ to the CAT gene in both orientations (pCB6-73A and pCB6-73B) and downstream of the 3′ non-coding region (pCB6-79A and pCB6-79B) (Figure 1). These constructs were then conjugated into *Agrobacterium tumefaciens* GV3111SE, which harbors a disarmed Ti-plasmid (pTiB6S3-SE) (Fraley *et al.*, 1985). Transformation of tobacco (*Nicotiana tabacum* cv. *Xanthi*) leaf discs and plant regeneration were carried out as described (Horsch *et al.*, 1985). Regenerated plants that were Kan^r were tested for the production of nopaline (Otten and Schilperoort, 1978).

Seed and embryo development in tobacco plants

Tobacco seeds reach maturity within 22−24 days after pollination in the greenhouse. Seeds increase in size during the first 10 days after pollination due to an increase in the size of the multinucleate endosperm, during which time embryos are in the globular stage (Figure 2). Beginning at day 10, embryos enlarge through heart, torpedo and cotyledon stages of development, followed by an increase in cotyledon cell size until seeds reach maturity. Tobacco seed storage proteins begin to accumulate 12 days after pollination (data not shown). Tobacco seed development differs from soybean seed development in several respects. First, tobacco seeds contain a large endosperm during early stages of development while soybean seeds contain a very small amount of endosperm. Second, soybean seed development occurs over a longer period of time (45−120 days depending upon the cultivar) compared to tobacco seeds; nevertheless, the α′-subunit gene is expressed during the corresponding developmental stage in each seed type, i.e. during mid-to-late stages of development (Naito *et al.*, submitted; Meinke *et al.*, 1981).

The expression of the CAT gene driven by the 35S promoter in transgenic plants

CAT enzyme activity was determined in extracts derived

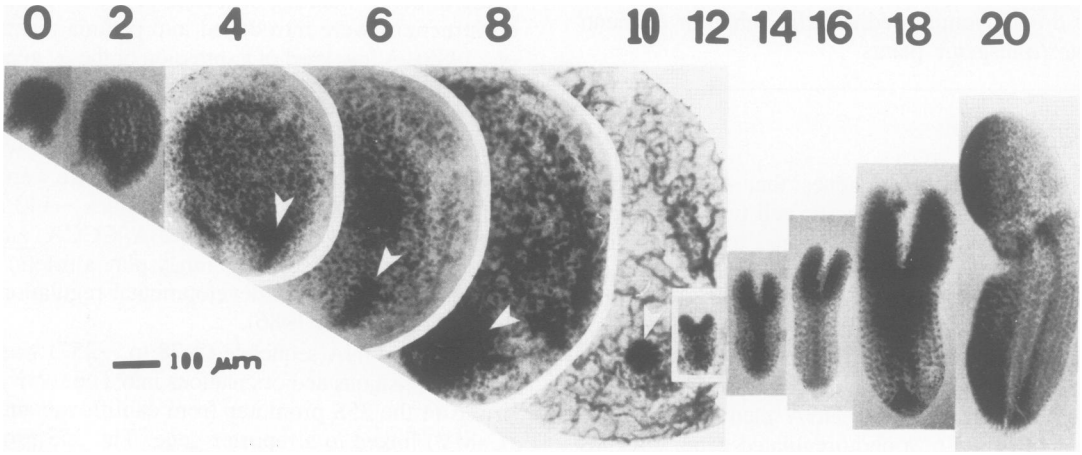

Fig. 2. Tobacco seed and embryo development. Transgenic tobacco plants were grown in the greenhouse and flowers were tagged upon pollination. Seed pods were harvested at different days post pollination. Seeds were collected and fixed with 3:1 ethanol (100%): acetic acid (glacial) solution. Seeds were stained by a Feulgen stain procedure (Sharm and Sharm, 1972). After dehydration, seeds and embryos were mounted and photographed.

from plant tissues as described in Materials and methods. Figure 3A presents the results of an assay using equivalent amounts of protein from different tissues of plants harboring pCB6-36 (Figure 1). Similar levels of CAT activity were detected in roots, stems and leaves of transgenic tobacco plants, although there was ~2-fold higher level of activity in younger tissues than in older tissues (data not shown). CAT activity was also assayed in extracts derived from seeds collected at different stages of development. As shown in Figure 3A the level of CAT activity appears to decrease in seeds during mid-to-late stages of development. This apparent decrease is due to the specific accumulation of tobacco seed storage protein but not of CAT during these stages, such that the ratio of CAT activity to total protein decreased. In contrast, when equivalent volumes of extracts prepared from equal numbers of seeds were used for the assay, CAT activity remained relatively constant after 13 days post pollination (dpp) (Figure 3B), a point after which there is not a significant increase in the number of cells per seed (Figure 2). The constant level of CAT activity per seed after 13 dpp relative to the CAT activity at day 8 provides a basal level that is convenient for measuring the effects of the α'-gene upstream sequence on the expression of the chimeric gene.

The upstream DNA sequence from the α'-gene promoter can act as an enhancer element

The 170-bp upstream DNA element from the α' gene was introduced into pCB6-36 at different positions and orientations (Figure 1) and the various gene constructs were introduced into tobacco plants. The levels of CAT activity were then measured in extracts of leaves, stems and roots, and in seeds from a number of different transgenic plant lines. The average level of CAT activities in those plants is shown in Table I.

Insertion of the upstream element of the α' gene into the

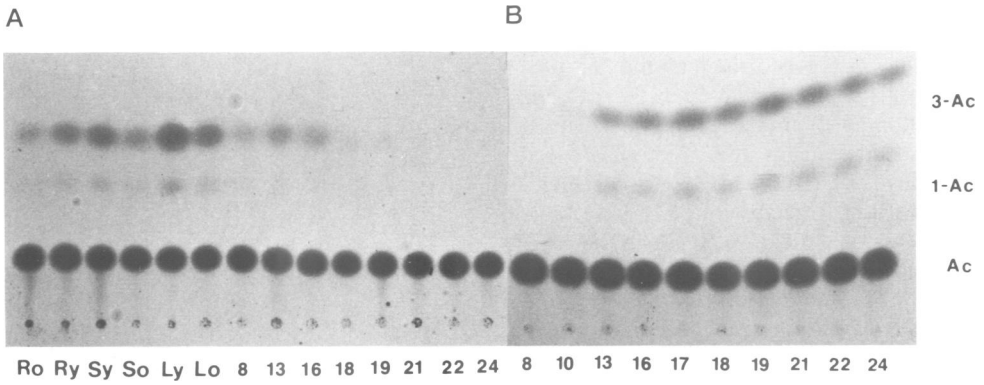

Fig. 3. CAT activity in tobacco plants harboring pCB6-36. CAT activity was assayed essentially as described (Gorman *et al.*, 1982). **A.** Results of assays that included equal amounts of protein (1 μg) extracted from roots, stems, leaves or seeds. **B.** CAT activity in assays in which protein extracted from 10 seeds were used. Abbreviations: Ry, Sy and Ly; young root, stem and leaf. Ro, So and Lo; older root, stem and leaf. Ac, 2-Ac and 3-Ac: [^{14}C]chloramphenicol, 2-, 3-acetylchoramphenicol.

Table I. Expression of CAT gene in transgenic tobacco plants
A. CAT activity in roots, stems and leaves[a]

Construct	No. of plants	Roots	Stems	Leaves
6-36	8	23 ± 4	26 ± 4	31 ± 5
6-37A	6	28 ± 6	21 ± 4	29 ± 6
6-37B	5	28 ± 5	28 ± 5	35 ± 7
6-73A	3	20 ± 7	19 ± 4	25 ± 7
6-73B	4	26 ± 6	26 ± 6	24 ± 7
6-79A	4	33 ± 9	30 ± 7	38 ± 9
6-79B	2	20 ± 6	23 ± 5	30 ± 8

B. Relative CAT activity in seeds at different day post pollination (dpp)[b]

Construct	No. of plants	8	10	12	14	16	18	20	22	24
6.36	8	1 ± 0	2 ± 0.3	3 ± 0.3	7 ± 0.7	11 ± 2	11 ± 1	11 ± 2	12 ± 1	11 ± 2
6-37A	6	1 ± 0	2 ± 0.3	3 ± 0.5	39 ± 5	264 ± 34	316 ± 63	502 ± 97	442 ± 83	435 ± 89
6-37B	5	1 ± 0	3 ± 0.3	5 ± 0.9	52 ± 10	289 ± 46	362 ± 67	422 ± 85	523 ± 115	513 ± 107
6-73A	3	1 ± 0	2 ± 0.2	4 ± 0.3	9 ± 3	22 ± 7	22 ± 6	24 ± 7	26 ± 7	26 ± 6
6-73B	4	1 ± 0	3 ± 0.5	4 ± 0.7	10 ± 3	27 ± 6	30 ± 5	28 ± 5	29 ± 6	28 ± 4
6-79A	4	1 ± 0	2 ± 0.5	3 ± 0.6	6 ± 1	8 ± 2	10 ± 2	11 ± 5	12 ± 4	12 ± 4
6-79B	2	1 ± 0	2 ± 0.6	2 ± 0.6	4 ± 2	7 ± 2	10 ± 3	11 ± 3	11 ± 5	12 ± 3

[a]The level of CAT activity is expressed as % conversion of chloramphenicol into its acetyl derivatives in extracts from roots, stems and leaves. The values indicated are average CAT activities from 2–8 independent transgenic plants. ± values indicate the standard errors.
[b]To calculate relative CAT activity, the level of CAT activity at each seed stage was compared with that at 8 days post pollination. Underlined values indicate the significant enhancement in relative CAT activity.

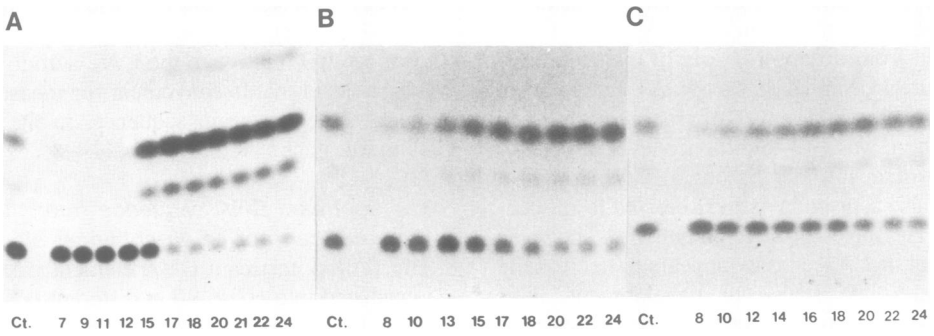

Fig. 4. CAT activity in tobacco plants harboring the chimeric gene containing the DNA element from the α′-gene promoter. An equal number of seeds from each stage of seed development was used for the CAT assays as described in Figure 3. **A, B** and **C**, enzyme activity in seeds of plants harboring, respectively, pCB6-37A, pCB6-73A and pCB6-79B (Figure 1). Ct, CAT activity at day 20 dpp in pCB6-36.

chimeric CAT gene did not affect its expression in leaves, stems, or roots of transgenic plants (Table IA). In seeds, however, there were significant differences in CAT enzyme activity between the various gene constructs. Figure 4A shows expression of the CAT gene driven by the 35S promoter into which the upstream element from the α′ gene was inserted at position −90 in the proper orientation (pCB6-37A). During early stages (8−12 dpp) of seed development, a low level of CAT activity was detected, similar to that in plants transformed with pCB6-36 (Table IB). However there was a signficant increase in the level of CAT activity in seeds from 14 dpp to maturity in plants harboring pCB6-37A. CAT activity increased by ~5-fold at day 14 and by at least 25-fold thereafter. Similar results were obtained when the upstream sequence was placed in the reverse orientation (pCB6-37B) (Table IB).

To verify that the increase in CAT activity reflected increased levels of gene expression, the level of CAT mRNA accumulation was examined in immature seeds from plants harboring pCB6-36 and pCB6-37A. As shown in Figure 5, the increase in CAT enzyme activity presented in Figure 4A and Table IB correlates with an increase in the amount of CAT mRNA.

To test whether the function of the enhancer element is dependent upon its position relative to the transcriptional start site, the element was placed 3′ of the CAT coding region (pCB6-73A, B; Figure 1). Figure 4 shows the levels of CAT gene expression in these transgenic plants. The level of CAT activity during early seed development was similar to that observed for a CAT gene without the element (pCB6-36), but in later stages of seed maturation (after 14 dpp), a reproducible, 2- to 5-fold enhancement of CAT activity was detected (Figure 4B). Similar results were obtained with transformants harboring pCB6-73B, in which the element is in the reverse orientation (Table IB).

When the upstream element was placed in either orientation 3′ to the polyadenylation region of the CAT gene (pCB6-79A, B; Figure 1), there were no detectable differences in the levels of CAT activity compared to control plants (pCB6-36) (Figure 4C, Table IB).

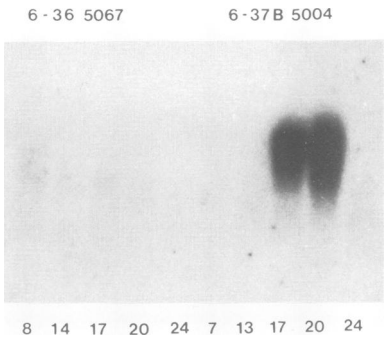

Fig. 5. Level of CAT mRNA in transgenic tobacco seed. Total RNA was isolated from tobacco seeds at increasing days post pollination. Ten micrograms of total RNA was separated in 1.2% agarose gels containing formaldehyde, blotted to a nitrocellulose filter and hybridized to a ^{32}P-labeled fragment from the coding region of the CAT gene. The numbers 5067 and 5004 refer to samples isolated from two independent transgenic plants.

Discussion

Timko *et al.* (1985) reported that an upstream regulatory sequence from a photoregulated gene, *rbcS* ss3.6, can direct a high level of photoregulated expression independent of orientation of the sequence relative to its homologous promoter, but this sequence did not function when placed 3′ to the TATA box or the transcriptional start site. We demonstrate here that the regulatory element from the α′ gene can function as a transcriptional enhancer when placed both 5′ to the transcriptional start site of the 35S promoter or 3′ to the coding region (Table I), but not when placed 3′ of the polyadenylation sequence. This element shows a positive regulatory function, in contrast with results reported recently (Kuhlemeier *et al.*, 1987) that describes a negative regulatory element in a photoregulated gene, *rbcS*-3A.

The enhancement of gene expression caused by insertion of the upstream element from the α′-gene promoter in the chimeric CAT gene occurred in mid-to-late stages of seed development (Figure 4). A similar developmental pattern of enhancement was observed when the element was placed in reverse orientation (pCB6-37B) and in the region 3′ to the CAT coding sequence (pCB6-73A, B), although the level of enhancement is substantially lower in the latter case (Table I). The developmental stages during which transcriptional enhancement occurs are those during which the polypeptides for the α′ gene start to accumulate in transgenic tobacco and

petunia (Beachy *et al.*, 1985; Naito *et al.*, in press) plants harboring the complete α′-subunit gene. These data suggest that temporal expression of the α′-subunit gene promoter is controlled by the sequences at −78 to −257 in the upstream region of the α′ promoter. However additional experiments are required to determine whether or not other sequences in the promoter have similar or additive activities.

In other experiments (Naito *et al.*, in press), the α′- and β-subunit genes of β-conglycinin were linked together in either direct (←,←) or divergent (←,→) orientations relative to their promoters, and transferred into petunia plants. A higher level of the β-gene expression was observed when the two genes were linked in the divergent orientation than when the β gene was introduced alone, or downstream of the α′ gene. However, the timing of the onset of expression of the two genes was independent of gene orientation. It is possible that the enhanced expression of the β gene resulted from the close proximity (~2 kb) of the enhancer element in the α′-gene promoter to the β-gene promoter. The lack of enhancement in constructs of the direct orientation may be due to the distance that separated the β-gene promoter from the enhancer element (>4 kb).

These experiments indicate that the strong *cis*-acting element located between −257 and −78 of the α′ gene responds to cellular conditions during specific stages of seed development, presumably by interacting with *trans*-acting factor(s) that are present during these stages, which regulate expression of the α′ gene. Other experiments are under way to investigate whether other regulatory elements are present in the 5′-flanking sequences of the α′ gene and to understand how each element might interact with *trans*-acting factors to control expression of this embryo-specific gene.

Materials and methods

Construction of the chimeric reporter gene and conjugation into A.tumefaciens

The DNA fragment containing the polyadenylation signal region was obtained by restricting the α′-subunit gene *Gmg*17.1 (Doyle *et al.*, 1986) with *Eco*RI and *Nde*I. The ends of the 450 bp DNA fragment released were made blunt by a fill-in reaction with the Klenow fragment of DNA polymerase. This DNA fragment contains sequences starting 110-bp downstream of the termination codon (TGA) of the α′-gene; its characteristics have been previously described (Schuler *et al.*, 1982). The intermediate plasmid used was pMON316 (Sanders *et al.*, 1987) which is a derivative of pMON200 (Fraley *et al.*, 1985) and contains the 35S promoter of CaMV with a polylinker region and the 3′-end from the nopaline synthase gene. The fragment was inserted in the proper orientation into the *Eco*RI and *Hin*dIII sites of pMON316 (Sanders *et al.*, 1987) that had been made blunt. A *Sau*3A fragment carrying the CAT coding sequence was isolated from pBR328 (Soberon *et al.*, 1980) and inserted into the *Bgl*II site of pMON316.

The upstream DNA element containing nucleotides −78 to −257 from the region 5′ of the α′ gene (Chen *et al.*, 1986) was subcloned and inserted in the 35S promoter at position −90 (at the *Eco*RV site in the promoter fragment) in both orientations (pCB6-37A,B). The DNA sequence element was also inserted 3′ to the coding sequence of the CAT gene and upstream of the 3′ non-coding region of the α′ gene in a filled-in *Xho*I site (pCB6-73A,B). For the constructs pCB6-79A,B, the element was inserted at a filled-in *Hin*dIII site within the fragment containing the α′-gene 3′ polyadenylation signal. These constructs were then conjugated into *A.tumefaciens* strain GV3111SE carrying a disarmed Ti plasmid (pTiB653-SE) (Fraley *et al.*, 1985). Transformation of tobacco leaves and regeneration of plants were carried out as described (Horsch *et al.*, 1985).

Assay for CAT activity in transgenic tobacco plants

Total protein extracts from roots, stems, leaves or seeds were prepared and CAT activity was assayed essentially as described (Gorman *et al.*, 1982). Reactions were carried out in 150 μl with 1 nmol of [^{14}C]chloramphenicol (0.05 μCi) for 15 min at 37°C. In assays in which protein from equal numbers of seeds were used, 200 seeds from each stage of tobacco seed development (day 8−24 post pollination) were ground in 400 μl of grinding buffer and 20-μl aliquots of the extracts were used in the reactions. Thin-layer chromatograms were exposed to X-ray film overnight. Radioactivity was quantitated in a liquid scintillation counter after cutting the appropriate spot from the thin-layer plates.

Isolation and analysis of RNA from developing seeds of transgenic tobacco plants

Total RNA was isolated from different stages of developing tobacco seeds removed from seed capsules as previously described (Chen *et al.*, 1986). Total RNA was resolved by electrophoresis in 1.5% agarose gels containing formaldehyde, blotted to nitrocellulose and hybridized to purified CAT gene coding sequences (*Bgl*II and *Xgo*I fragment from pCB6-36; indicated in Figure 1) labelled with ^{32}P by nick translation. The filter was hybridized in a solution containing 0.75 M NaCl, 75 mM sodium citrate, pH 7.2 and 40% formamide at 42°C overnight, washed at 42°C in 0.15 M NaCl, 15 mM sodium citrate containing 0.1% SDS for 1 h, and exposed to X-ray film.

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