

Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter

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Mutations affecting spatial and temporal regulation of a β -phaseolin gene encoding the major storage protein of bean (*Phaseolus vulgaris*) were analyzed by stable and transient transformation approaches. The results substantiate the value of transient assays for rapid determination of the functionality of *cis*-acting sequences and the importance of stable transformation to identify tissue-specific determinants. Spatial information is specified primarily by two upstream activating sequences (UAS). UAS1 (–295 to –109) was sufficient for seed-specific expression from both homologous and heterologous (CaMV 35S) promoters. *In situ* localization of GUS expression in tobacco embryos demonstrated that UAS1 activity was restricted to the cotyledons and shoot meristem. A second positive domain, UAS2 (–468 to –391), extended gene activity to the hypocotyl. Temporal control of GUS expression was found to involve two negative regulatory sequences, NRS1 (–391 to –295) and NRS2 (–518 to –418), as well as the positive domain UAS1. The deletion of either negative element caused premature onset of GUS expression. These findings indicate combinatorial interactions between multiple sequence motifs specifying spatial information, and provide the first example of the involvement of negative elements in the temporal control of gene expression in higher plants.

Key words: *cis*-acting domains/embryo development/gene expression/seed storage proteins/transient assays

Introduction

The development of higher plant seeds is a striking example of a highly orchestrated developmental process. Investigation of regulatory constraints on the expression of seed storage proteins provides an excellent opportunity to learn how positional and temporal information is decoded into patterns of gene activity by eukaryotic cells. Changes in the abundance and diversity of mRNA sequences during embryogenesis in soybean (Goldberg *et al.*, 1981a) and cottonseed (Galau and Dure, 1981) were initially demonstrated by DNA–RNA hybridization studies. Later, cloned sequences were used as probes to follow changes in the abundance of individual mRNAs during the early, mid

and late stages of embryogenesis (Goldberg *et al.*, 1981b; Murray and Kennard, 1984; Barker *et al.*, 1988; Hughes and Galau, 1989). Storage protein genes are expressed only during the mid-maturation phase of development, after cell division and morphogenesis are essentially complete (Crouch, 1987). During this phase, reserve materials in the form of proteins, starch and lipids accumulate primarily in the expanding cotyledons and to a lesser extent in the embryonic axis. Phaseolins, the major storage proteins of the French bean, display this type of regulation (Sun *et al.*, 1978). Northern analysis (Murray and Kennard, 1984) demonstrated the temporal regulation of phaseolin mRNA levels, which reach a peak when the cotyledons are 17 mm in length. Very little or no phaseolin protein (Sun *et al.*, 1978; Mutschler *et al.*, 1980) or mRNA (Murray and Kennard, 1984) occur in vegetative organs or very young (cotyledons <5 mm) embryos. Organ and cell-specific expression of genes encoding major storage proteins from legumes has been studied for the proteins themselves (Greenwood and Chrispeels, 1985; Sengupta-Gopalan *et al.*, 1985; Bray *et al.*, 1987) and the corresponding mRNAs (Barker *et al.*, 1988; Perez-Grau and Goldberg, 1989). These studies showed that accumulation of storage proteins and their mRNAs is confined to the cotyledons and the embryonic axis (excluding the root meristem). Developmentally regulated expression of bean phaseolin in embryos of transgenic tobacco seed revealed the evolutionary conservation of regulatory and processing mechanisms for mRNA (transcription and intron splicing) and seed storage protein (transit signal cleavage and correct glycosylation). These findings have subsequently been confirmed for bean lectin and many other seed protein genes from legumes, *Brassica*, *Arabidopsis* (Guerche *et al.*, 1990) and sunflower in a variety of transgenic plants (reviewed by Goldberg *et al.*, 1989).

Bustos *et al.* (1989) showed that phaseolin 5'-flanking sequences (–795 to +20) are sufficient to confer correct spatial and temporal regulation upon a bacterial gene coding for the enzyme β -glucuronidase (GUS) in transgenic tobacco plants. Similarly-regulated expression of reporter genes has been demonstrated for upstream sequences from soybean β -conglycinin (Chen *et al.*, 1988), corn zein (Scherthner *et al.*, 1988) and barley β -hordein (Marris *et al.*, 1988). Therefore, although post-transcriptional regulation of phaseolin mRNA stability can affect the level of phaseolin accumulation (Chappell and Chrispeels, 1986), primary control of the time and location of phaseolin expression is at the level of gene transcription.

The complexity of the phaseolin upstream region was indicated by our (Bustos *et al.*, 1989) demonstration that an upstream A/T-rich sequence (–682 to –628) enhances GUS expression from a minimal CaMV 35S promoter in many vegetative tissues of tobacco but, when present in the context of the full-length phaseolin promoter, is silent in all tissues other than the developing embryo. By means of gene transfer

experiments in tobacco and transient gene expression assays in bean cotyledon cells, we now show that the β -phaseolin upstream region (-795 to +20) has a modular organization of at least five regulatory domains that activate or decrease GUS expression *in vivo*. It appears that no single domain exhibits all the properties of the entire region; rather, the several domains interact to confer seed-specificity, regional distribution within embryonic tissues and developmental control.

Results

Functional mapping of cis-acting domains in the phaseolin upstream region

Nested and internal deletions were made throughout the phaseolin -795 to +20 region and the resulting sequences were fused upstream of the translation initiation codon of a reporter β -glucuronidase gene from *Escherichia coli* (Figure 1). All phaseolin sequences were cut at the *ScaI* site (+20) and fused to the *SmaI* site of the promoterless GUS vector pBI201.1, a pUC19-based plasmid with a GUS-*nos3'* cassette identical to that in pBI101.1 (Jefferson, 1987). Consequently, correct initiation of transcription by the phaseolin promoter resulted in mRNAs that included the first 20 nucleotides of the phaseolin 5' untranslated leader. Deletion end-points corresponded to positions -795, -518, -468, -418, -391, -295 and -109 (see Materials and methods).

These gene constructs were tested by transfection of bean cotyledon protoplasts and by gene transfer into tobacco plants. For transfection assays, the 6–10 mm cotyledon stage was chosen as the best compromise between phaseolin gene activity, cell size and starch content. Although steady-state levels of phaseolin mRNA are higher later in development, the greater cell size and starch

content reduce protoplast viability to unacceptably low levels. Details of the protocol carried out to optimize the conditions for efficient electroporation of plasmid DNA into cell protoplasts (Materials and methods) will be presented elsewhere (M.M.Bustos, M.J.Battraw and T.C.Hall, in preparation). A nearly linear relationship between GUS activity and supercoiled plasmid p795 concentration (up to 50 $\mu\text{g/ml}$) was obtained (data not shown). Even small quantities of input DNA (< 10 $\mu\text{g/ml}$) yielded GUS activity substantially above background levels. GUS activity was found to increase for the first 24 h after electroporation, followed by a plateau between 24 and 48 h. These experiments (data not shown) were conducted in the presence of a constant concentration (20 $\mu\text{g/ml}$) of a 35S/CAT plasmid (Odell *et al.*, 1985) as a control for the efficiency of DNA uptake by the cells. CAT activity assays (not shown) were reproducible within a given protoplast preparation. Differences were observed between protoplasts obtained at different seasons and from seeds at different stages of development. All gene constructs shown in Figure 1, except p518(d391/295), were stably integrated into the genome of tobacco plants by Ti-plasmid-mediated transformation (see Materials and methods). Eight to nine independently-transformed plants exhibiting normal morphologies, high seed fertility and Kan^R phenotype were recovered in each case. The number and structure of integrated transgenes were analyzed by Southern hybridization of tobacco leaf genomic DNA with a GUS probe, and by segregation of the Kan^R phenotype (data not shown). In every case, a minimum of six independent transformants with a low number of integrated gene copies (1–3) were selected for further analysis of GUS specific activity in mature seeds.

Transient versus stable GUS expression from the various phaseolin promoter constructs are compared in Figure 2A. Each cross-hatched bar on the left represents the average

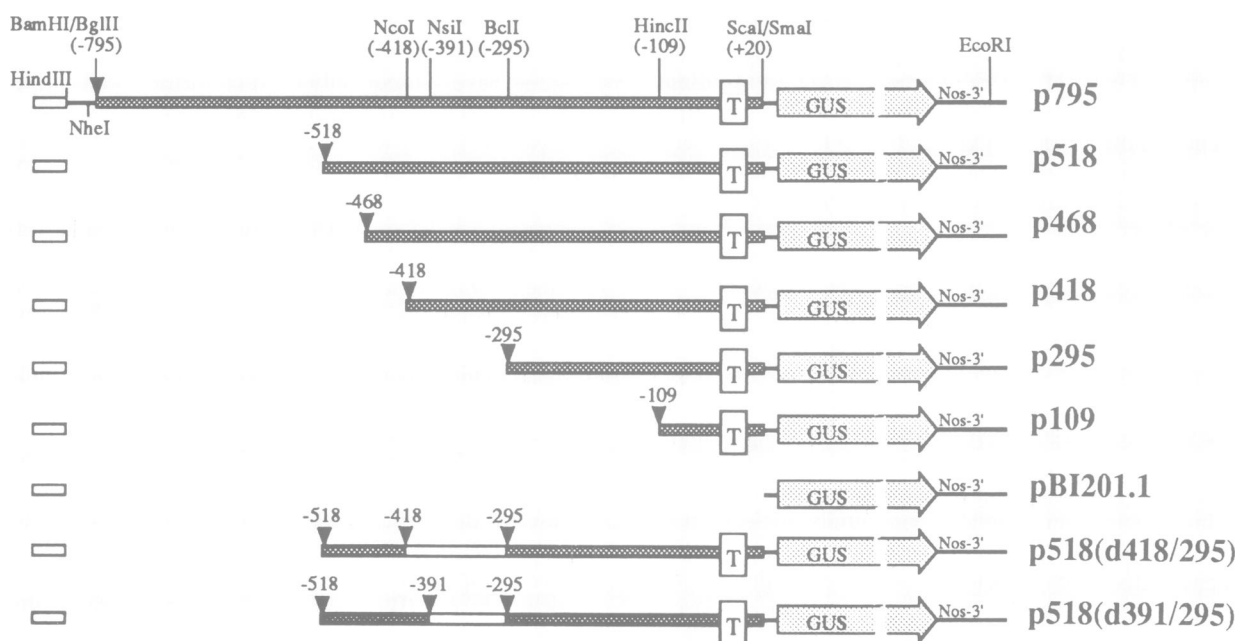


Fig. 1. Diagram of phaseolin-GUS gene fusions. Nested and internal deletions were made in the phaseolin -795 to +20 region and later inserted upstream of the GUS-*nos3'* cassette in the vector pBI201.1 (Jefferson, 1987). The 3'-end of all phaseolin sequences (■) corresponds to the same *ScaI* site. Nucleotide positions of deletion end-points are indicated with numbered arrowheads. Internal deletions in constructs p518(d418/295) and p518(d391/295) are symbolized by empty boxes between deletion end-point markers. □ Duplicated TATA boxes.

of at least five transfection experiments with independent bean protoplast preparations. Solid bars on the right represent average GUS specific activities in mature seed (35 mg or ~ 500 seeds) from each group of plants harboring the same construct. In order to compare the relative effects of upstream mutations on gene activity in each experimental system, all measurements were normalized to the value corresponding to construct p795, shown as 100% (dotted lines). The diagram in Figure 2B depicts the location of phaseolin upstream DNA domains as deduced from these analyses.

Constructs p518 and p468 displayed essentially the same activity in protoplasts and in transgenic seeds. Apparent differences in average values were not statistically significant in either case. In contrast, the deletion of 50 bp between positions -468 and -418 (construct p418) caused a 65–70% reduction in transient GUS expression in bean

protoplasts relative to construct p518. A similar reduction was observed in tobacco seeds. It is apparent from these data that positions -468 and -418 encompass part of an upstream activating sequence (UAS), indicated by UAS2 in Figure 2B. Further deletion of the fragment between -418 and -295 (construct p295) caused a net increase in activity in both systems. This result is especially important because it provides evidence for the presence of a negative regulatory sequence (NRS1 in Figure 2B) between positions -418 and -295. Additional support for the existence of a down-regulating sequence was obtained by analysis of internal deletions in the -418 to -295 region. An *Nsi*I–*Bcl*I DNA fragment (positions -391 to -295, Figure 1) was removed from plasmid p518 to produce construct p518(d391/295). This construct yielded a higher GUS activity in bean transient assays (225%) than p518 (Figure 2A, left panel), which was

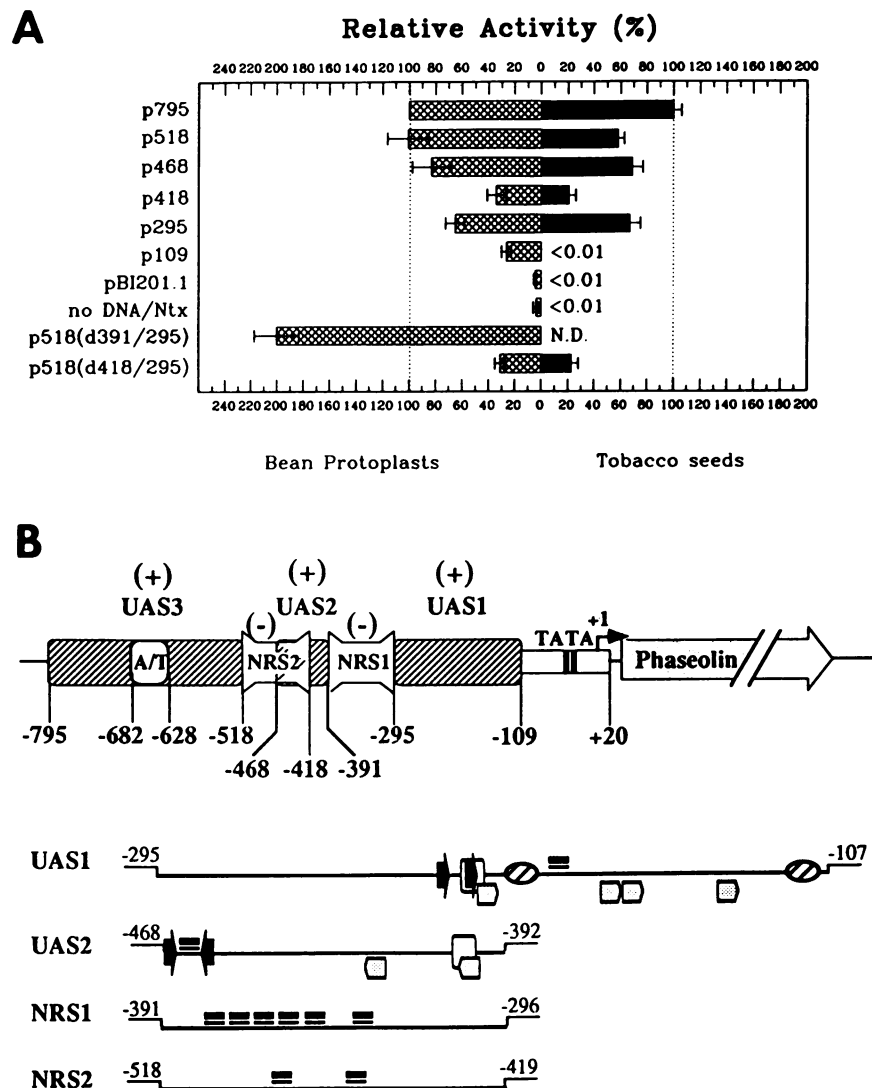


Fig. 2. (A) Comparison of relative activities of phaseolin–GUS fusion constructs in transiently transfected bean protoplasts and in mature seeds of stably transformed tobacco. Bars represent the average activity for each gene construction, either from multiple transfections (cross-hatched bars on the left), or from multiple (minimum of six) independently transformed tobacco plants (solid bars on the right). All values are presented as the percentage relative to construct p795 (100%, dotted lines). ‘no DNA/Ntx’ refers to electroporation with no plasmid DNA added on side A, and wild-type non-transformed tobacco seeds on side B. N.D.: not done. Construct p518(d391/295) was tested in bean protoplasts only. Construct designations are as in Figure 1. (B) Diagram of the phaseolin upstream DNA region. *Cis* domain boundaries were defined functionally by deletion experiments shown in (A). The signs in parentheses indicate the relative effect of each domain on GUS expression *in vivo*. The approximate locations of conserved sequence motifs are indicated in the diagrams at the bottom. ■, ACC trinucleotides; □, soybean β -conglycinin AACCCA motif (Allen *et al.*, 1989); □, CCACC/A motif; ⊗, soybean AACACA motif (Goldberg, 1986); ■, AGAAC/AA.

consistent with the difference observed between constructs p418 and p295. This experiment confirmed the function of the -391 to -295 region (NRS1) as a negative regulatory sequence and suggested that the 3' boundary of positive domain UAS2 was located upstream of nucleotide -391 . Further deletion of 27 bp from construct p518, between positions -418 and -391 , yielded plasmid p518(d418/295), which had only 30% of the activity of the p518 in bean protoplasts and 20% in transgenic seeds. This nearly

Table I. Organ specificity of GUS expression in tobacco

	Leaf	Root	Mature seed
Phaseolin promoter			
p109	5 ± 0	1 ± 0	19 ± 1
p295	2 ± 0	5 ± 0	13774 ± 625
p519(d418/295)	2 ± 0	40 ± 1	4671 ± 259
p418	2 ± 0	21 ± 3	4354 ± 250
p468	3 ± 0	40 ± 1	14229 ± 463
p518	4 ± 0	46 ± 1	11943 ± 392
p795	3 ± 0	96 ± 4	20550 ± 630
Hybrid promoters			
pUAS1/35S	3 ± 0	8 ± 2	16669 ± 969
p35SE/109	11695 ± 890	17590 ± 702	1127 ± 12
p35SE/295	8362 ± 249	4138 ± 124	13203 ± 387
pBI120, Ntd	3 ± 0	6 ± 1	45 ± 1

Average GUS specific activities (pmol 4-MU/min/mg protein) are listed together with the SE. Measurements were done on 35 mg of mature (dry) seeds and in leaves and roots from 21-day-old tobacco seedlings germinated and grown in the presence of 400 µg/ml kanamycin. Ntx: non-transformed *Nicotiana tabacum* cv. Xanthi.

10-fold decrease relative to the NRS1 deletion construct p518(d391/295), the largest difference seen between any two consecutive deletions, strongly supported the idea that the 27 bp fragment contains a necessary portion of domain UAS2. Consequently, the region -468 to -295 includes a positive transcriptional element (UAS2), closely followed by a negative one (NRS1).

The reduced activity of construct p518(d418/295) relative to p295, in both systems, revealed the presence of a second negative domain (NRS2, Figure 2B) between nucleotides -518 and -418 . In fact, construct p518(d418/295) corresponds to the replacement of domain NRS1, in plasmid p418, with domain NRS2. These two constructs yielded almost identical relative activities in both systems, indicating that the two negative domains are functionally equivalent, i.e. they repress the activity of the -295 to $+20$ region by a similar amount.

Deletion of 186 bp from the 5'-end of p295 produced the minimal promoter construct p109 (Figure 1) that yielded lower GUS activity than p295 in both transient and transgenic assays. This suggested the existence of yet another positive element, indicated as UAS1 on Figure 2B. In bean protoplasts, construct p109 yielded a significant amount of GUS expression ($\sim 20\%$ of p795) above the background value ($< 3\%$) of GUS-like activity measured when no DNA was supplied to the electroporation cuvette. The first 109 bp upstream of the transcription initiation site, the phaseolin minimal promoter region, contain two TATA boxes (at -32 and -43) and a putative CCAAT box at -78 . GUS expression with construct p109 probably reflects the ability of the phaseolin minimal promoter region to promote basal levels of transcription in transfected protoplasts. This

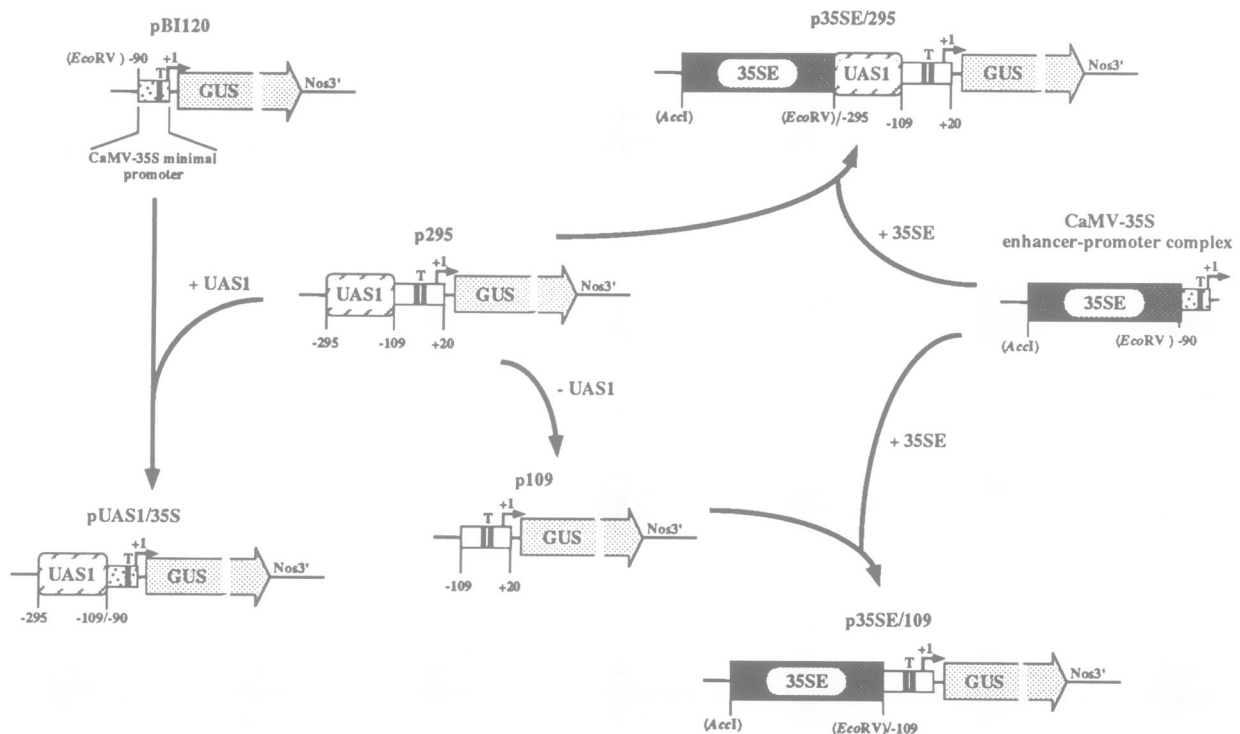


Fig. 3. Diagrams of hybrid promoters combining phaseolin and CaMV 35S promoter elements. Phaseolin-GUS constructs p295 and p109 are the same as those shown in Figure 1. Labeled arrows indicate the sources of fragments used in the construction of each hybrid promoter. \square symbolizes the phaseolin minimal promoter region as defined in this study (see text).

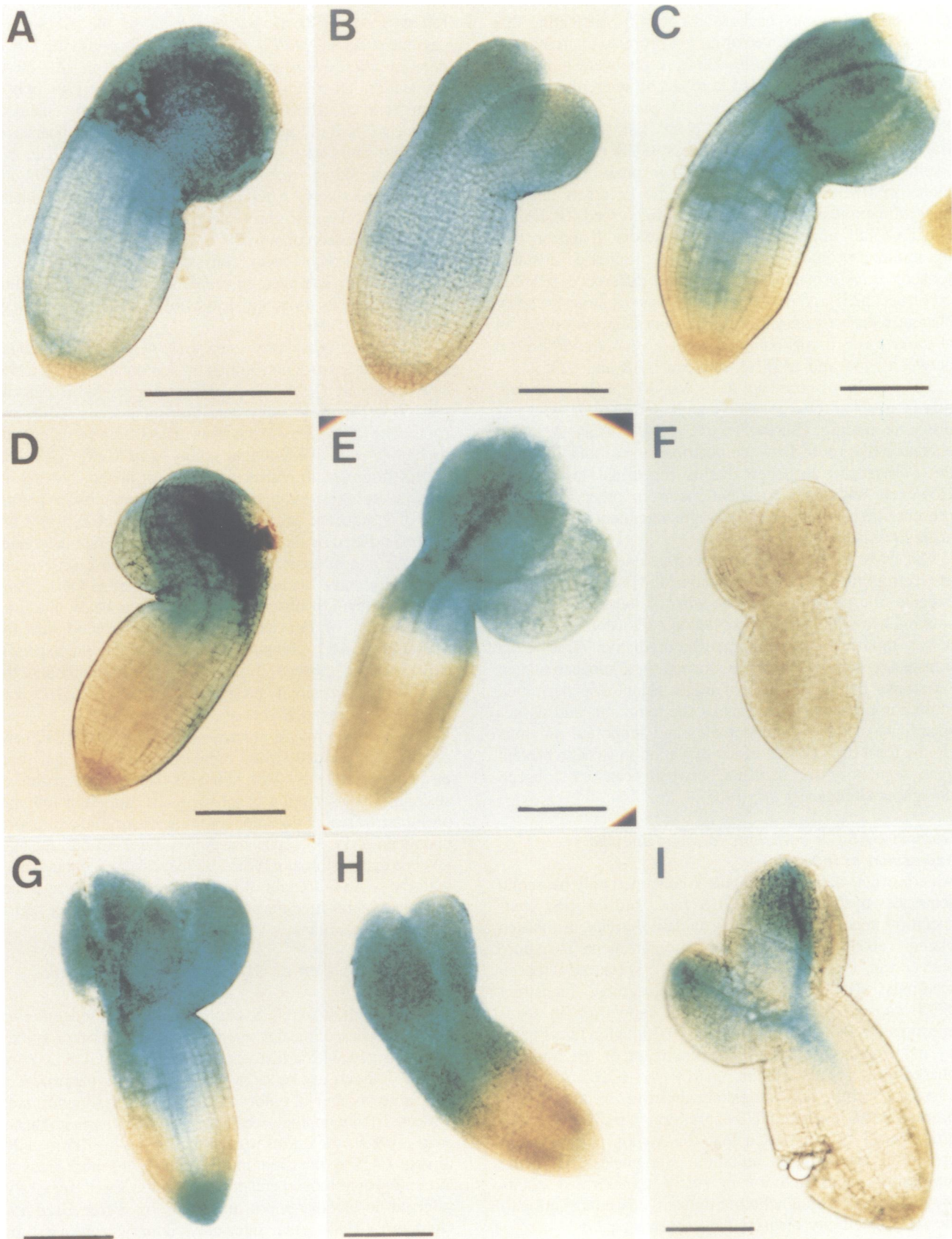


Fig. 4. Histochemical localization of GUS activity in transgenic tobacco seed embryos. Bars = 200 μ m. A, p795; B, p518; C, p468; D, p418; E, p295; F, p109; G and H, pUAS1/35S; I, p35SE/109.

view is supported by the fact that the promoter-less construct pBI201.1 yields only background activity, indicating that DNA sequences in the vector do not promote expression of the GUS gene in bean cells. These results were in marked contrast with those obtained in tobacco, where the level of GUS (or GUS-like) activity in p109 plants was similar to those seen in non-transformed plants (Ntx) and in plants transformed with the promoterless GUS construct. An identical deletion (−295 to −109) in phaseolin genomic clone pPhV3.8 also resulted in the complete loss of phaseolin expression in transgenic tobacco seeds (R.Klassy and T.C.Hall, unpublished data). Therefore, it appears that the minimal promoter region is largely repressed in stable tobacco transformants. Although the difference between constructs p295 and p109 in tobacco was the largest relative change seen between any two adjacent deletion points, in absolute terms it was only 40% greater than the difference between p468 and p418 or between p418 and p295.

The activity of construct p518 was 40–50% lower than that of p795 in tobacco. This result is consistent with our previous finding (Bustos *et al.*, 1989) of an A/T-rich enhancer-like motif between positions −682 and −628 of the β -phaseolin sequence that is functional in tobacco. However, additional contributions from other sequences present in the −795 to −518 fragment cannot be ruled out at this point.

The above analyses indicated that transient gene expression in electroporated protoplasts from bean cotyledon cells can expedite the mapping of *cis*-regulatory sequences that are active during the phase of cotyledon expansion. However, it was important to corroborate the predictive value of the transient system by stable transformation of tobacco plants. As shown below, the use of intact plants also permitted more comprehensive analysis of the temporal- and tissue-specificity of the various domains and supported previous claims for a high degree of conservation in storage protein gene regulatory mechanisms between bean and tobacco (Sengupta-Gopalan *et al.*, 1985).

Domain UAS1 is sufficient for seed-specific expression in tobacco

All constructs depicted in Figure 1 contained only phaseolin sequences upstream of the GUS gene, and directed seed-specific expression of β -glucuronidase activity in tobacco plants. Table I lists GUS activity measurements in mature seeds, and in leaves and roots of 3-week-old tobacco seedlings. The shortest phaseolin fragment (construct p295) capable of yielding abundant expression in seeds encompasses the phaseolin −295 to +20 region (see Figure 1) and contains domain UAS1 upstream of the minimal phaseolin promoter region (−109 to +20). Although construct p109 was completely inactive in tobacco, it promoted low levels of GUS expression in transfected bean protoplasts. This indicated that this region is capable of promoting transcription initiation. Nonetheless, complete repression of its activity in stably transformed plants prevented establishing whether it makes any contribution to the seed-specificity displayed by construct p295.

Consequently, three new hybrid promoters were made which incorporated phaseolin and CaMV 35S *cis*-acting sequences. Figure 3 illustrates the relationships between different hybrid and non-hybrid promoter sequences. Plasmids p295 and p109 are identical to those in Figure 1.

Plasmid pBI120 contains the CaMV 35S minimal promoter region from −90 to +6, upstream of the GUS-*nos3'* cassette. Plasmid pUAS1/35S contains the phaseolin UAS1 domain (*BclI*–*HincII* fragment) inserted in the direct orientation upstream of pBI120. The other two hybrid promoters, p35SE/109 and p35SE/295, have the enhancer fragment (35SE in Figure 5) from the CaMV 35S enhancer–promoter complex (*AccI*–*EcoRV* fragment, Odell *et al.*, 1985) inserted upstream of phaseolin constructs p109 and p295, respectively. After being subcloned into the binary vector pBIN19, the three new constructs were used to transform tobacco plants. Six to eight plants independently transformed with each construct were obtained and characterized. The average of GUS activity measurements in seeds, and in roots and leaves of 3-week-old seedlings are listed in Table I.

Of the three hybrid promoters, only pUAS1/35S exhibited seed-specific expression similar to that obtained with p295. The truncated CaMV 35S promoter present in plasmid pBI120 yielded near background activity in seeds, leaves and roots. The low activity in the roots differs from recent reports by Benfey *et al.* (1989) on the ability of the CaMV 35S −90 to +8 fragment to enhance expression in the root tips of tobacco embryos and in the roots of mature tobacco plants. The 35S sequence present in plasmid pBI120 differs from the one utilized by these investigators in several nucleotides near the site of transcription initiation. These differences apparently make it a less efficient promoter in tobacco and lower the level of un-enhanced expression below the limit of detection of our technique. We concluded that the phaseolin UAS1 domain is sufficient for seed-specific expression in tobacco, both from the phaseolin and the CaMV 35S minimal promoters (constructs p295 and pUAS1/35S, respectively).

The other two hybrid promoter constructs, p35SE/109 and p35SE/295, yielded strong expression in leaves and roots in addition to seeds. This indicates that, while capable of sustaining transcription in tobacco, the phaseolin minimal promoter fragment does not confer seed-specific expression upon the CaMV 35S enhancer region. Furthermore, the high level of expression in p35SE/295 leaves and roots indicates that the UAS1 domain does not contain any sequences capable of abolishing the activity of the 35S enhancer region in vegetative organs.

Spatial distribution of GUS expression in tobacco embryos

The distribution of GUS expression in excised embryos harboring phaseolin deletion constructs and hybrid promoters was analyzed with a histochemical stain for GUS activity. Figure 4 shows examples of the predominant patterns seen in a large number of embryos (20 from each transformant) dissected from mature seeds. As reported earlier (Bustos *et al.*, 1989), the entire regulatory region (−795 to +20) directs GUS expression in the cotyledons and areas of the embryonic axis corresponding to the shoot apex (also referred to as the shoot meristem by Perez-Grau and Goldberg, 1989), the inter-cotyledonary zone and the hypocotyl (the region between the embryonic shoot and root meristems). The only regions of p795 embryos devoid of GUS activity were the root meristem and the root cap (panel A). The same distribution of GUS activity was observed upon deletion of sequences down to position −468

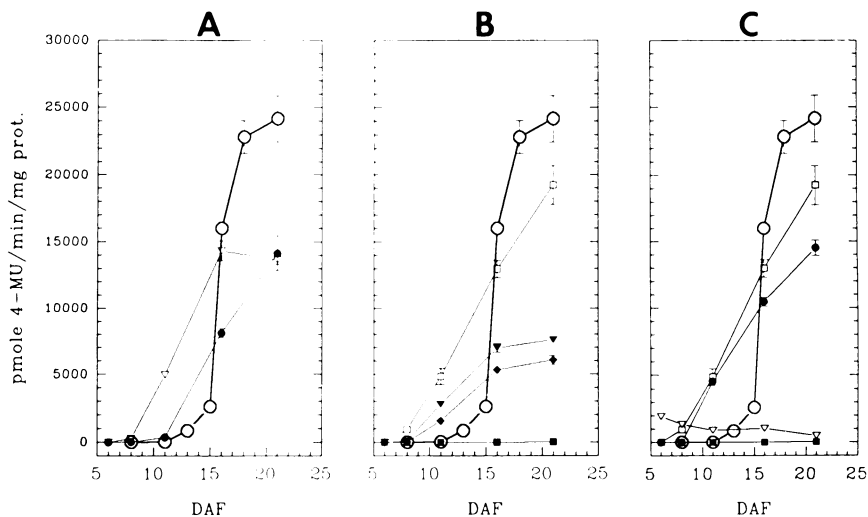


Fig. 5. Temporal regulation of GUS expression in developing tobacco seeds. Curves illustrate changes in average specific activities as a function of time in days after flowering (DAF). Panels **A** and **B**: gene constructs containing only phaseolin sequences fused upstream of the GUS gene. \circ , p795; ∇ , p518; \bullet , p468; \blacktriangledown , p418; \square , p295; \blacklozenge , p518(d418/295); \blacksquare , p109. Panel **C**: hybrid promoters. \bullet , pUAS1/35S; ∇ , p35SE/109. Data from p795 and p295 plants are reproduced for comparison. ∇ , pBI120.

(constructs p518 and p468 in panels B and C, respectively). In contrast, the deletion between positions -468 and -418 (p418, panel D), which inactivated the upstream positive domain UAS2, resulted in loss of GUS expression in the hypocotyl region of the embryonic axis. The remaining GUS activity could be seen in the cotyledons and the shoot apex. A similar distribution was found in p295 embryos (E), which displayed a sharp boundary approximately perpendicular to the hypocotyl–root axis and located a few cell layers below the inter-cotyledonary zone. As predicted by the very low values obtained from fluorescence assays, no histochemical staining was apparent in p109 embryos (F) even after prolonged incubation times. Two alternative patterns of GUS localization were observed with the hybrid promoter pUAS1/35S. The first one (G), seen in $\sim 60\%$ of the embryos, showed strong activity in the cotyledons, the shoot apex and in the root meristem and root cap. The elevated expression in the root is most likely due to a root-specific enhancer sequence located between positions -90 and -46 of the 35S minimal promoter fragment (Lam *et al.*, 1989). Since embryos transformed with pBI120 showed no detectable GUS activity in the root meristem, we hypothesize that expression in that tissue of pUAS1/35S embryos is the result of synergistic interactions between *cis* elements in the phaseolin UAS1 domain and domain A of the CaMV 35S promoter (Benfey *et al.*, 1989). The second pattern (H) is essentially identical to the one seen in p295 embryos (E). Interestingly, GUS activity in p35SE/109 embryos was concentrated primarily in the procambium (the precursor of the vascular tissues) of the embryonic cotyledons and the upper portion of the axis. Although some diffuse staining can also be seen in the epidermis and storage parenchyma of the cotyledons, it is much lower than the activity seen for p295 or pUAS1/35S in the same cell types.

From the histochemical localization of GUS activity, we conclude that domain UAS1 is responsible for most or all of the activity of the phaseolin regulatory region in the embryonic cotyledons. Additional expression in the hypocotyl of the axis requires the presence of the second positive domain UAS2.

Temporal regulation during seed development: involvement of the negative domains NRS1 and NRS2

The physiological importance of co-ordinating the onset of storage protein synthesis with the emergence and differentiation of storage organs has been emphasized by the decreased stability of β -conglycinin subunits in early stages of soybean seed development (Shattuck and Beachy, 1985). Phaseolin accumulation commences 8–10 days after flowering (DAF) in bean (Sun *et al.*, 1978) and 11–13 DAF in tobacco seeds (Sengupta-Gopalan, 1985). This ‘off–on’ switching is concomitant with the expansion of the cotyledons and the start of endogenous storage protein synthesis (Sano and Kawashima, 1983). Regulated and intense induction is also apparent for the GUS gene under the control of the phaseolin -795 to $+20$ region in tobacco (Bustos *et al.*, 1989). The convenience and accuracy of GUS measurements (as opposed to immunological detection of phaseolin polypeptides on protein blots) gave us an opportunity to examine in detail the temporal regulation of gene expression by phaseolin promoter sequences, with special emphasis on onset and subsequent induction. GUS expression in seeds containing the various phaseolin and chimeric promoter constructs (Figures 1 and 3) was monitored between 6 and 21 DAF (Figure 5), at times specifically chosen to compare relative specific activities before and after the critical 11 DAF stage. In order to minimize developmental variation, all plants were grown in the same chamber under controlled conditions of temperature, humidity and illumination, and seeds were collected during the same time of the year. The accumulation of endogenous tobacco storage proteins was monitored by SDS–PAGE and staining with Coomassie Brilliant blue, in the same extracts used for GUS activity measurements (data not shown). The appearance of the prominent and characteristic bands representing the subunits of the 12S storage globulins (Sano and Kawashima, 1983) between the 11 and 16 DAF stages, provided a convenient developmental reference point. The profile of GUS accumulation in plants transformed with construct p795 (heavy lines, large open circle) is shown for comparison in panels A, B and C of Figure 5. This reveals that, until day

11, p795 seeds contained only background levels of GUS activity (<50 units). Subsequently, GUS levels rose sharply being 500–600 times higher by day 16. Seeds harboring construct p518 (panel A) displayed a similar temporal regulation but yielded ~50% lower GUS levels.

The onset of GUS expression appeared to be advanced by 3 days (to 8 DAF) in seeds containing construct p468, which lacks the NRS2 domain but still contains domains UAS2 and NRS1. This temporal shift was reflected at 11 DAF (Figure 5, panel A) by a 25- to 100-fold higher activity relative to p795 or p518 seeds. Maximum activity was evident in p518 and p468 seeds between 18 and 21 DAF. As found previously in mature seeds (Table I), little difference was detected between these two constructs at 21 DAF. Construct p418 (which lacks domains NRS2 and UAS2, but contains NRS1) and p295 (with neither), both shown in Figure 5B, also had elevated levels of expression at 11 DAF. Furthermore, construct p518(d418/295) which has domain NRS2 in place of UAS1, displayed the same effect. This indicates that both negative domains NRS1 and NRS2 are required to delay onset of expression until 11 DAF.

As shown in Figure 5B, GUS expression from construct p295, which has only the positive domain UAS1, begins between 8 and 11 DAF and reaches a maximum between 18 and 21 DAF. To determine whether this profile of induction is characteristic of the seed-specific UAS1 domain or general for the period of development between 8 and 21 DAF, the time-course of GUS accumulation in p295 seeds was compared to those in seeds containing the hybrid promoters pUAS1/35S and p35SE/109. As illustrated in Figure 5C, the two hybrid promoters yielded very different profiles of expression. While pUAS1/35S expressing GUS activity between 8 and 11 DAF and showed a similar induction to that observed with construct p295, construct p35SE/109 directed expression throughout the entire 6 to 21 DAF period. Furthermore, GUS activity in p35SE/109 seeds at 6 DAF was at least 100 times higher than in any of the other constructs analyzed and decreased by ~50% at 21 DAF. These results indicated that domain UAS1 is sufficient for the large induction seen with the phaseolin deletion construct p295.

We concluded from these experiments that the temporal regulation displayed by the phaseolin upstream region (–795 to +20) involves two main components: intense induction by positive domain UAS1 that starts between 8 and 11 DAF and reaches a maximum between 18 and 21 DAF; and negative regulation by the region comprised between nucleotides –518 and –295, which delays the onset of expression and requires the presence of both negative domains NRS1 and NRS2.

Discussion

The data presented here demonstrate that the upstream region of a bean β -phaseolin gene contains multiple *cis*-acting domains regulating gene transcription by the promoter. The ability of these domains to modulate the intensity of expression of a GUS reporter gene was initially delineated by transient expression in bean cotyledon protoplasts. These effects were confirmed using stably transformed tobacco

plants, which also permitted analysis of their role in spatial and temporal regulation.

Transient assays have been widely used to study gene regulation in animal cells and in protoplasts from monocots and dicots (Werr and Lörz, 1986; Ellis *et al.*, 1987; Lipphardt *et al.*, 1988; Jones *et al.*, 1989; Logemann *et al.*, 1989). They permit rapid tests of DNA mutations and are not subject to genome position effects (Schell, 1987; Gidoni *et al.*, 1988). Examples of accurate gene regulation in plant transient assay systems include UV-induction of gene expression from the chalcone synthase promoter in parsley protoplasts (Dangl *et al.*, 1987), the ABA-induction of Em expression in protoplasts from cultured rice cells (Marcotte *et al.*, 1988) and the induction of gene expression from a maize *Adh-1* promoter by low O₂ concentrations in maize protoplasts (Walker *et al.*, 1987).

A pre-condition for the activity of transfected gene constructs containing the phaseolin promoter is the availability of *trans*-acting factors capable of sustaining phaseolin specific transcription. In bean plants of the cultivar Tendergreen, expression of phaseolin mRNAs begins when the cotyledons are 6–8 mm in length, and is strong in the 10–11 mm stage used in our experiments. Our conclusion that GUS expression in cotyledon cells reflects specific interactions between cotyledon cellular *trans*-acting factors and DNA sequences present in the transfected DNA was supported by a number of experimental results. For example, the amount of GUS expression in cotyledon cells was a function of the amount of input DNA and was entirely dependent on the presence of phaseolin upstream sequences (Figure 2A). Furthermore, a comparison of transient GUS expression in protoplasts from immature cotyledons or hypocotyls of 8-day-old bean seedlings (M.M.Bustos *et al.*, in preparation), showed that p795 yields 10- to 20-fold higher expression levels in the cotyledon protoplasts than does a full-length 35S promoter (pBI221: Jefferson, 1987). This indicates that cotyledon protoplasts contain similar *trans*-acting factor(s) to those in intact cotyledon. Notably, the close correspondence for results from transient and stable expression assays strongly supports the value of the protoplast system for mapping *cis*-acting elements.

In some situations, however, transient and stable expression experiments have led to different conclusions concerning the activity of specific DNA sequences. Using transgenic tobacco plants, Fang *et al.* (1989) found that the region between nucleotides –343 and –208 of the CaMV 35S promoter was responsible for ~40–50% of the total activity in leaf tissues. In contrast, Ow *et al.* (1987) determined that the same region had no apparent effect on the amount of luciferase expressed in transiently-transformed carrot protoplasts. A region downstream of position –208 was necessary for expression in both experimental systems. Similarly, our results indicate that the phaseolin –795 to –518 region (UAS3) appears to increase expression in stably transformed tobacco seeds but has no effect on transient expression levels (Figure 2A). Since histochemical localization of GUS activity has shown that an A/T-rich element present in that region is considerably more active in the axis than in the cotyledons of tobacco embryos (Bustos *et al.*, 1989), the apparent lack of effect of the –795 to –518 deletion in bean transient assays may be explained by the fact that axial tissues were manually removed prior

to the preparation of protoplasts (Materials and methods). Alternatively, UAS3 function could require a particular chromatin conformation only attainable when the gene is stably integrated at a genomic location (Stief *et al.*, 1989).

Although very useful for delineating DNA domains that modulate the intensity of gene expression in a particular cell preparation or developmental stage, transient assays have limited ability to yield information on tissue specificity and temporal regulation. Consequently, comparative analysis of GUS expression in seeds, leaves and roots of transgenic tobacco was undertaken. This revealed that all deletion constructs containing only phaseolin sequences upstream of the reporter gene yielded seed-specific expression. The presence in domain UAS1 (186 nucleotides) of *cis*-acting elements regulating spatial expression was evident from the high levels of seed-specific activity observed when this domain was placed upstream of either the minimal phaseolin (p295) or the 35S (pUAS1/35S) promoter fragments. The 340-fold activation of gene expression by UAS1 for both these promoters is much higher than the 25- to 40-fold enhancement of CAT activity reported (Chen *et al.*, 1988) in tobacco seeds for a soybean β -conglycinin gene fragment inserted within a full-length 35S promoter–enhancer complex. While the data of Chen *et al.* (1988) do not exclude the possibility of interactions between the soybean DNA sequences and the 35S enhancer region that was present in all their constructs, our experiments reveal that the UAS1 domain confers the intensity of expression, spatial distribution and temporal regulation seen for the –295 to +20 region. This conclusion is supported by our assessment of the contribution of the phaseolin proximal promoter region (–109 to +20) to organ specificity. Construct p35SE/109, containing the 35S enhancer fragment fused upstream of phaseolin position –109, yielded high levels of expression in leaves and roots as well as in seeds, suggesting that the proximal region behaves as a spatially neutral promoter element. All seed-specific constructs yielded GUS expression in the endosperm as well as in embryonic tissues (M. Bustos and T. Hall, in preparation).

Studies on the localization of mRNAs from Kunitz trypsin inhibitor (*Kti*) genes in soybean (Perez-Grau and Goldberg, 1989) and 2S albumin (*at2S*) genes in *Arabidopsis* embryos (Guerche *et al.*, 1990) by *in situ* hybridization, have revealed that at least one member of each gene family (*Kti3* and *at2S1*, respectively) is expressed specifically in the embryonic hypocotyl. The remaining genes (*Kti1*, *Kti2*, *at2S2*, *at2S3* and *at2S4*) are expressed throughout the embryonic axis and cotyledons in a way similar to that of the major storage globulin genes. Previously, phaseolin expression has been shown to occur in the axis and cotyledons of bean embryos (Mutschler *et al.*, 1980) and of transgenic tobacco embryos (Greenwood and Chrispeels, 1985). The results presented here on histochemical analysis of the distribution of GUS activity in mature tobacco embryos (shown in Figure 4), are important in showing that distinct *cis*-acting DNA sequences confer spatially-regulated activation of gene expression within different regions (i.e. cotyledons, shoot apex and hypocotyl) and in different cell types of the same region (i.e. parenchyma and procambium) of the embryos. For example, the activity of domain UAS1 was localized in the cotyledons and the shoot apex but expression in the hypocotyl requires the presence of a second activating domain, UAS2 (Figure

4, compare panels C and D). Additionally, although both domain UAS1 and the 35S enhancer fragment directed GUS expression in the cotyledons and shoot apex, UAS1 yielded much higher activity in storage parenchyma cells (Figure 4, compare panels E and I) and the 35S enhancer was mostly active in the procambial strands.

Mechanistically, it can be postulated that cell-type or organ-spatial regulation derives from interactions of different *trans*-acting factors with specific recognition sites in each *cis*-acting domain. An example could be the different cell type-specificities of UAS1 and the 35S enhancer in the cotyledons. Alternatively, cell-specificity could result from similar *cis* elements appearing in different combinatorial arrangements (Yamamoto *et al.*, 1985) in each gene promoter, thereby leading to dissimilar interactions with a given set of *trans*-acting factors common to all cell types. In this regard, it is interesting to note the presence within domains UAS1 and UAS2 of an assortment of repeated, short sequence motifs conserved in seed-expressed genes from related legumes (Figure 2B). For instance, there are six copies of the pentameric sequence CCAC_A^C (four in UAS1 and two in UAS2), a single copy of the 40 nucleotide-long ‘vicilin-box’ sequence (Gatehouse *et al.*, 1986) in UAS1, and two copies of the hexamer AACACA (Goldberg, 1986) also in UAS1. Even more remarkable is the occurrence of nine copies of the motif AGAA_A^CA, six of which are concentrated within domain NRS1 (Figure 2B). Undoubtedly, small motifs such as these are combinatorial participants in the regulatory matrix. How these elements interact and where the precise delineations of the functional regions lie will require fine-resolution mapping through the analysis of expression of many more constructs.

Gene expression in seeds containing either constructs p795 or p518 first occurred between 11 and 13 DAF, namely, 3 days later than for constructs p295 and pUAS1/35S. The earlier onset (8–11 DAF) and subsequent intensification of expression seen for the latter constructs appeared to be specifically associated with the presence of domain UAS1 (e.g. the 35S enhancer construct p35SE/109 did not display the same regulation), and probably reflects changes in the abundance or activity of *trans*-acting factor(s) that interact with this domain. Early activation was also seen when different combinations of the domains NRS2, UAS2 and NRS1 were placed upstream of p295. Examples of that are constructs p468 (domains UAS2 and NRS1), p418 (domain NRS1 alone), p518(d391/295) (domains NRS2 and UAS2) and construct p518(d418/295) (domain NRS2 alone). Thus, the –518 to –295 region appears to contain elements modulating the temporal activity of p295. Additionally, the maximum level of expression was also dependent on the balance of positive and negative elements present.

While the role of the negative elements in repressing gene activity prior to 11 DAF is clear, the contribution of positive domain UAS2 to temporal regulation is less obvious. It is possible that, in addition to its role as a gene activator in the hypocotyl, UAS2 also reverses the repressing activity of the negative elements after 11 DAF, thereby restoring gene expression to the value set by domain UAS1. The negative effect of domains NRS1 and NRS2 appears to be limited to the onset and total level of expression, since the spatial distribution of GUS activity was the same irrespective of their presence or absence. A similar distinction between

factors specifying cell-specific activation of transcription and those responsible for its temporal modulation, has been made in connection with lineage-specific gene expression in the sea urchin (Davidson, 1989).

Materials and methods

Plasmid constructions and plant transformations

Plasmid constructions used for tobacco transformants and bean transient expression assays are depicted schematically in Figure 1. Clone p795, the same as p β +20/GUS (Bustos *et al.*, 1989), consists of an 815 bp *Hind*III–*Sca*I restriction fragment from clone pVPh3.8 (Sengupta-Gopalan *et al.*, 1985), subcloned into the GUS vector pBI101.1 (Jefferson, 1987). Deletion constructs p518 and p468 were obtained by the *Exo*III–S1 nuclease procedure (Erase-a-Base, Promega) starting at the site for *Nhe*I in clone p795. To construct deletions p418, p295 and p109, DNA restriction fragments (*Nco*I–*Sca*I, *Bcl*I–*Sca*I and *Hinc*II–*Sca*I) from phaseolin genomic clone pVPh3.8 were first subcloned into pUC19. In a second step, these fragments were inserted into the polylinker region of the GUS vectors pBI101.1 and pBI201.1 between sites for *Hind*III and *Sma*I. Consequently, phaseolin DNA sequences in all 5'-nested deletion constructs have identical 3' ends, corresponding to nucleotide position +20. Constructs p518(d391/295) and p518(d418/295) were created by inserting *Hind*III–*Nsi*I and *Hind*III–*Nco*I fragments from clone p518, upstream of clone p295. The hybrid promoter constructs p35SE/109 and p35SE/295 shown in Figure 3 were made by ligating the enhancer region (*Ac*cI–*Eco*RV) from the CaMV 35S promoter present in plasmid pBI221.1 (Jefferson *et al.*, 1987), upstream of clones p109 and p295, respectively. The hybrid promoter construct pUAS1/35S was made by subcloning a *Bcl*I–*Hinc*II fragment from clone p795 into the polylinker region of vector pBI120 (a gift from R.Jefferson; see Bustos *et al.*, 1989) which contains a CaMV 35S promoter truncated at position –90 upstream of the GUS gene. All gene constructs were subcloned into the binary vector pBIN19 (Bevan, 1984) prior to tobacco transformation by the leaf-disc method of Horsch *et al.* (1985). Regeneration of transformed tobacco plants was carried out as previously described (Bustos *et al.*, 1989).

Transient expression assays in bean cotyledon protoplasts

Seeds of the common bean (*P. vulgaris*) variety 'Bush Bean Tendergreen' were purchased from L.L.Olds Seed Co. (Madison, WI). The seeds were surface sterilized in 10% bleach, 0.05% Triton X-100 at room temperature for 10 min, rinsed with water and planted on 1:1 vermiculite:peat moss. Plants were grown in a greenhouse at 76–80°F. Protoplasts were obtained by an adaptation of the technique of Frearson *et al.* (1973). Seed pods were harvested 10–12 days after flowering. Immature seeds were removed and surface sterilized in a beaker with 10% bleach, 0.05% Triton X-100 for 10 min. After thorough washing with sterile distilled water the seeds were placed in CPW 13M (Frearson *et al.*, 1973) and the cotyledons were dissected out and separated from the axis. Only cotyledons between 6 and 11 mm in length were used in subsequent steps. After mincing with a scalpel, the cotyledon tissue (~2 g) were placed in 50 ml of a solution containing 1% Cellulase RS (Yakult Honsha Co. Ltd, Tokyo, Japan) and 0.1% Pectolyase Y23 (Seishin Pharmaceutical Co. Ltd, Tokyo, Japan) in CPW 13M and incubated at 28°C for 4 h. Cell protoplasts were released by filtering the tissues through two Nytex filters (250 and 80 μ m mesh size) and washing with an equal volume of CPW 13M medium. Protoplasts were harvested by centrifugation (55 g, 5 min) and washed once each in CPW 13M and HBS 427 (10 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM CaCl₂ and 427 mM mannitol). Protoplasts were resuspended to a density of 2×10^5 viable cells/ml (as determined by fluorescein diacetate staining) in HBS 427, mixed with supercoiled plasmid DNA (e.g. 40 μ g) and electroporated as described by Fromm *et al.* (1987). Protoplasts were allowed to recover for 10 min at room temperature before mixing with 2 ml of KPR medium (Kao, 1977) containing 100 μ g/ml carbenicillin. Cells were incubated in small (35 \times 10 mm) culture dishes at 28°C in the dark, typically for 36 h, prior to assaying for GUS activity (Jefferson, 1987).

DNA extractions and Southern blot analysis

Genomic DNA was isolated from leaf tissues by the method of Taylor and Powell (1982). Southern blot analysis was performed according to Maniatis *et al.* (1982) using a GUS DNA probe (500 bp, *Eco*RV–*Eco*RV restriction fragment from pBI201.1) labeled by the random-primed method (Boehringer-Mannheim) in the presence of [³²P]dCTP. The number of gene copies inserted in each transformant was estimated from the number and intensity of hybridizing bands present on blots of single restriction endonuclease digests.

Quantification of GUS specific activity and histochemical in situ localization in tobacco seed embryos

GUS activities in seeds, leaves and roots were measured as previously described (Bustos *et al.*, 1989). Batches of 35 mg of seeds were collected at various stages of development and extracts were prepared for triplicate fluorimetric assays using the substrate 4-methyl-umbelliferyl glucuronide. Protein concentrations were estimated using the colorimetric assay of Bradford (1976). Data on GUS activities, protein concentrations and gene copy numbers were maintained and analyzed using an *ad hoc* IBM-PC GUS Data Management Program (M.Bustos, unpublished data). *In situ* localization of GUS activity in seed embryos and endosperm was carried out as described in Bustos *et al.* (1989).

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References

- Allen, R.D., Bernier, F., Lessard, P.A. and Beachy, R.N. (1989) *Plant Cell*, **1**, 623–632.
- Barker, S.J., Harada, J.J. and Goldberg, R.B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 458–462.
- Benfey, P.N., Ren, L. and Chua, N.-H. (1989) *EMBO J.*, **8**, 2195–2202.
- Benfey, P.N., Ren, L. and Chua, N.-H. (1990) *EMBO J.*, **9**, 1677–1684.
- Bevan, M.W. (1984) *Nucleic Acids Res.*, **12**, 8711–8721.
- Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
- Bray, E.A., Naito, S., Pan, N.S., Anderson, E., Dubé, P. and Beachy, R.N. (1987) *Planta*, **172**, 364–370.
- Bustos, M.M., Guitinan, M.J., Jordano, J., Begum, D., Kalkan, A. and Hall, T.C. (1988) *Plant Cell*, **1**, 839–853.
- Chappell, J. and Chrispeels, M.J. (1986) *Plant Physiol.*, **81**, 50–54.
- Chen, Z.-L., Pan, N.-S. and Beachy, R.N. (1988) *EMBO J.*, **7**, 297–302.
- Crouch, M.L. (1987) In Browder, L.W. (ed.), *Developmental Biology: A Comprehensive Synthesis*. Plenum Press, New York, Vol. 5, pp. 367–404.
- Dangl, J.L., Hauffe, K.D., Lipphardt, S., Hahlbrock, K. and Schell, D. (1987) *EMBO J.*, **6**, 2552–2556.
- Davidson, E.H. (1989) *Development*, **105**, 421–445.
- Domoney, C. and Casey, R. (1987) *Planta*, **170**, 562–566.
- Ellis, J.G., Llewellyn, D.J., Dennis, E.S. and Peacock, W.J. (1987) *EMBO J.*, **6**, 11–16.
- Fang, R.-X., Nagy, F., Sivasubramanian, S. and Chua, N.-H. (1989) *Plant Cell*, **1**, 141–150.
- Frearson, E.M., Power, J.B. and Cocking, E.G. (1973) *Dev. Biol.*, **33**, 130–137.
- Fromm, M., Callis, J., Taylor, L.P. and Walbot, V. (1987) *Methods Enzymol.*, **153**, 351–366.
- Galau, G.A. and Dure, L.S. (1981) *Biochemistry*, **20**, 4169–4178.
- Gatehouse, J.A., Evans, I.M., Croy, R.R.D. and Boulter, D. (1986) *Phil. Trans. R. Soc. Lond. B*, **314**, 367–384.
- Gidoni, D., Bond-Nutter, D., Brasio, P., Jones, J., Bedbrook, J. and Dunsmuir, P. (1988) *Mol. Gen. Genet.*, **211**, 507–514.
- Goldberg, R.B. (1986) *Phil. Trans. R. Soc. Lond. B*, **314**, 343–353.
- Goldberg, R.B., Hoschek, G., Tam, S.H., Ditta, G.S. and Breidenbach, R.W. (1981a) *Dev. Biol.*, **83**, 201–217.
- Goldberg, R.B., Hoschek, G., Ditta, G.S. and Breidenbach, R.W. (1981b) *Dev. Biol.*, **83**, 218–231.
- Goldberg, R.B., Barker, S.J. and Perez-Grau, L. (1989) *Cell*, **56**, 149–160.
- Greenwood, J.S. and Chrispeels, M.J. (1985) *Plant Physiol.*, **79**, 65–71.
- Guerche, P., Tire, C., Grossi de Sa, F., De Clercq, A., Van Montagu, M. and Krebbers, E. (1990) *Plant Cell*, **2**, 469–478.
- Hagerman, P.J. (1990) *Annu. Rev. Biochem.*, **59**, 755–781.
- Hoffman, L.M. and Donaldson, D.D. (1985) *EMBO J.*, **4**, 883–889.
- Horsch, R., Fry, J., Hoffman, N., Eichholtz, D., Rogers, S. and Fraley, R. (1985) *Science*, **227**, 1229–1231.
- Hughes, D.W. and Galau, G.A. (1989) *Genes Dev.*, **3**, 358–369.
- Jefferson, R.A. (1987) *Plant Mol. Biol. Rep.*, **5**, 387–405.
- Jones, H., Ooms, G. and Jones, M.G.K. (1989) *Plant Mol. Biol.*, **13**, 503–511.
- Jofuku, D.K. and Goldberg, R.B. (1989) *Plant Cell*, **1**, 1079–1093.
- Kao, K.N. (1977) *Mol. Gen. Genet.*, **150**, 225–230.
- Kuhlemeier, C., Green, P.J. and Chua, N.-H. (1987) *Annu. Rev. Plant Physiol.*, **38**, 221–237.

- Lam, E., Benfey, P.N., Gilmartin, P.M., Fang, R.-X. and Chua, N.-H. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7890–7894.
- Lipphardt, S., Bretschneider, R., Kreuzaler, F., Schell, J. and Dangl, J.L. (1988) *EMBO J.*, **7**, 4027–4033.
- Logemann, J., Lipphardt, S., Lörz, H., Hauser, I., Willmitzer, L. and Schell, J. (1989) *Plant Cell*, **1**, 151–158.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Marcotte, W.R., Jr., Bayley, C.C. and Quatrano, R.S. (1988) *Nature*, **335**, 454–457.
- Marris, C., Gallois, P., Copley, J. and Kreis, M. (1988) *Plant Mol. Biol.*, **10**, 359–366.
- Murray, M.G. and Kennard, W.C. (1984) *Biochemistry*, **23**, 4225–4232.
- Mutschler, M.A., Bliss, F.A. and Hall, T.C. (1980) *Plant Physiol.*, **65**, 627–630.
- Odell, J.T., Nagy, F. and Chua, N.-H. (1985) *Nature*, **313**, 810–812.
- Ow, D.W., Jacobs, J.D. and Howell, S.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4870–4874.
- Perez-Grau, L. and Goldberg, R.B. (1989) *Plant Cell*, **1**, 1095–1109.
- Sano, M. and Kawashima, N. (1983) *Agric. Biol. Chem.*, **47**, 1305–1310.
- Schell, J. (1987) *Science*, **237**, 1176–1183.
- Scherthauer, J.P., Matzke, M.A. and Matzke, A.J.M. (1988) *EMBO J.*, **7**, 1249–1255.
- Sengupta-Gopalan, C., Reichert, N.A., Barker, R.F., Hall, T.C. and Kemp, J.D. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3320–3324.
- Shattuck-Eidens, D.M. and Beachy, R.N. (1987) *Plant Physiol.*, **78**, 895–898.
- Slightom, J.L., Sun, S.M. and Hall, T.C. (1983) *Proc. Natl. Acad. Sci. USA*, **82**, 3320–3324.
- Stief, A., Winter, D.M., Strätling, W.H. and Sippel, A.E. (1989) *Nature*, **341**, 343–345.
- Sun, S.M., Mutschler, M.A., Bliss, F.A. and Hall, T.C. (1978) *Plant Physiol.*, **68**, 918–923.
- Taylor, B.H. and Powell, A.L. (1982) *Focus*, **4**, 4–6.
- Walker, J.C., Howard, E.A., Dennis, E.S. and Peacock, W.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6624–6628.
- Werr, W. and Lörz, H. (1986) *Mol. Gen. Genet.*, **202**, 471–475.
- Yamamoto, K.R. (1985) *Annu. Rev. Genet.*, **19**, 209–252.

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