The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns

Philip N.Benfey, Ling Ren and Nam-Hai Chua

Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Ave., New York, NY 10021, USA

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We have analyzed expression conferred by two domains from the cauliflower mosaic virus (CaMV) 35S promoter and found different patterns in seeds, seedlings and seven week old plants. Expression from domain A (–90 to +8) is strongest in the radicle of the embryo, the radicle pole of the endosperm and in root tissue of seedlings and mature plants. Expression from domain B (–343 to –90) is strongest in the cells adjacent the cotyledon of the endosperm, in the cotyledons of the embryo and seedlings and in the leaves and stem of mature plants. When both domain A and domain B are present expression is detectable in most tissues at all stages of development. Thus analysis of a constitutive promoter in transgenic plants can be used to identify *cis* elements that confer tissue specific and developmentally regulated expression. **Key words:** 35S/developmental regulation/enhancer/histochemical localization/tissue specific

Introduction

The cauliflower mosaic virus (CaMV) 35S promoter has been shown to be highly active in most plant organs and during most stages of development when integrated into the genome of transgenic plants (Nagy *et al.*, 1985; Odell *et al.*, 1985; Jensen *et al.*, 1986; Jefferson *et al.*, 1987; Kay *et al.*, 1987; Sanders *et al.*, 1987). The 35S promoter can also confer expression in protoplasts of both dicots and monocots (Fromm *et al.* 1985; On-Lee *et al.*, 1986; Nagata *et al.*, 1987; Ow *et al.*, 1987; Odell *et al.*, 1988). In theory, expression from a constitutive promoter could be regulated by the interaction of *cis*-elements with factors that are present in all cell types. Alternatively, a constitutive promoter could contain multiple *cis*-elements which interact with different factors in different cell types.

Analysis of expression from the 35S promoter in floral tissue indicated the possible presence of multiple *cis*-elements (Benfey and Chua, 1989). In addition we have shown recently that a factor found in extracts of tobacco tissue can bind to a *cis*-element located between –90 and –59 of the 35S promoter. Mutation of four base pairs (bp) within this *cis*-element greatly reduced binding *in vitro* (Lam *et al.*, 1989). *In vivo* these mutations caused a large decrease in expression in root (E.Lam, P.Benfey, P.Gilmartin, R.X.Feng and N.-H.Chua, submitted). A 21 bp fragment containing this binding site was sufficient to confer expression in root when placed between the TATA box and the upstream region of the small subunit of the ribulose

bisphosphate carboxylase (*rbcS*) 3A gene from pea which normally expresses only in green tissue (E.Lam *et al.*, submitted). Additional evidence that this region is involved in expression in root tissue came from the observation that CAT enzyme activity was detected only in roots of transgenic plants that contained the 35S –90 to +8 region fused to the CAT coding sequence (Poulson and Chua, 1988).

These results suggested that the 35S promoter may contain at least two domains, one that confers expression principally in roots, the other that confers expression in other tissues. In these previous studies total RNA or CAT enzyme activity from entire organs of mature plants was measured. Here we show that a fragment from –90 to +8 can confer an expression pattern in transgenic plants that is markedly different from that conferred by a fragment from –343 to –90. We use histochemical localization to define the expression pattern of these two domains at the cellular level. In addition we analyze expression throughout development. Analysis of expression at certain stages of development provides clues as to the possible functional role of the *trans* factors that interact with the *cis*-elements under study.

Results

Constructs

We divided the 35S promoter into two domains: domain A (–90 to +8) and domain B (–343 to –90). Construct 1 contains domain A alone (Figure 1). Preliminary experiments indicated that deletion of domain A to –72 resulted in a complete loss of detectable expression. Therefore, we used construct 2 which contains the fragment from –72 to +8 as a negative control. Construct 3 contains domain B (–343 to –90) inserted upstream of the –72 to +8 fragment. Since no expression was detectable from construct 2 alone we postulated that expression from construct 3 would

Fig. 1. Constructs containing domain A and domain B of the 35S upstream region. Promoter fragments were ligated to the β -glucuronidase coding sequence as transcriptional fusions.

be due principally to domain B. Construct 4 contains both domain A and domain B, the -343 to -90 fragment inserted upstream of -90 to $+8$. The β -glucuronidase (GUS) coding sequence (Jefferson *et al.*, 1987) was placed downstream of all four constructs in such a way as to make a transcriptional fusion. We made transgenic plants that contained each of these constructs and analyzed expression of the GUS reporter gene in the progeny of the primary transformants.

Expression in mature seeds

Seeds were harvested from at least 8 independent transgenic plants containing each construct. Fresh sections were made by imbedding the seeds in an adhesive (see methods) and cutting 100 to 200 micron sections. These sections were then incubated with the histochemical substrate.

In mature seeds expression from domain A (construct 1) was localized to the radicle in the embryo and to the endosperm cells at the radicle pole (Figure 2A). This expression pattern was observed in 6 of 10 plants analyzed, the others showed no detectable expression. Expression in specific cells of the endosperm was unexpected since, to our knowledge, no biochemical or morphological difference among endosperm cells of tobacco has been previously reported (see for example, Avery, 1933). To rule out diffusion of enzyme or dye from embryo to endosperm during incubation as the cause of the endosperm staining we removed the embryo prior to incubation with the substrate. We again observed staining in the endosperm localized to the radicle pole (Figure 2B). In contrast, no staining in embryo or endosperm was observed in seeds from 16 independent transgenic plants containing construct 2 (-72 to $+8$).

Seeds that contain domain B (construct 3), showed expression principally in the cotyledons of the embryo and in the cells of the endosperm that are adjacent to the cotyledons (Figure 2C). This staining pattern was observed in eight transgenic plants. In two others in which staining was quite strong in the cotyledons, light staining at the tip of the radicle was also observed. Expression from domains A + B (construct 4) was detected in both the cotyledon and radicle of the embryo and in the regions adjacent to the cotyledon and radicle of the endosperm (Figure 2D) in seeds from eight plants.

We conclude that in mature seeds, the 35S promoter can be divided into two functional regions, one from -90 to $+8$ which is sufficient to confer expression in the radicle of the embryo and in the endosperm cells at the radicle pole, and the other from -343 to -90 that confers expression in the cotyledons and in the endosperm cells adjacent to the cotyledons. The division is not absolute; when there is high level expression in the cotyledons from the -343 to -90 fragment, there is also low level expression in the radicle.

Expression in seedlings

Seeds were sterilized and germinated on media containing the antibiotics, kanamycin and carbenicillin. Since all four constructs contain the neomycin phosphotransferase (NPTII)

coding sequence driven by the nopaline synthetase promoter, selection for plants containing the transgene should occur in media that contains kanamycin. We removed seedlings at 6, 10 and 17 days after planting. Tobacco seeds do not germinate synchronously (Avery, 1933), so the developmental stage of all seedlings was not precisely the same. The seedlings were pressed between glass slides in the presence of the histochemical substrate, then incubated with the substrate.

At 6 days, most seedlings containing domain A showed no detectable GUS expression. In 2 of the 10 plants analyzed, expression was detected in the root (Figure 2E). In seedlings containing domain B strong staining of the cotyledons was evident, as well as staining of the stele (or vascular tissue) in the hypocotyl and, in some plants, light staining at the root tip (Figure 2F). With domains A + B there was strong expression in both root and cotyledons, as well as staining in the stele and in other cells of the hypocotyl (Figure 2G). Seedlings with construct 2 showed no expression in any tissue (Figure 2H).

At 10 days, expression from domain A was detected in eight plants with the strongest staining localized to the root (Figure 2I). Staining in the root was most intense at the tip, in the root cap, in the epidermis and in root hairs. Seedlings at this stage containing domain B, showed expression in the root restricted to the vascular tissue and in a few plants, some expression at the tip of the root (Figure 2J). Plants with domains A + B showed expression throughout the root (Figure 2K). Plants with construct 2 showed no expression in the root (Figure 2L) or in any other tissue.

In addition to the predominant staining pattern in the root from domain A we consistently observed light staining just below the apical meristem (Figure 3A). Two plants containing this construct also showed light staining in the vascular tissue of the cotyledon. In seedlings containing domain B, staining was strongest in the vascular tissue of the hypocotyl, and there was no apparent staining just below the apical meristem (Figure 3B). In the cotyledons, staining was quite strong in the vascular tissue and in mesophyll cells (Figure 3C). In seedlings containing domains A + B both vascular tissue and the region just below the apical meristem stained in the hypocotyl and expression was strong in vascular and mesophyll tissue of the cotyledons (unpublished data).

At approximately 15–17 days lateral roots begin to form (Avery, 1933). In 17 day old seedlings containing domain A staining was strongest in the lateral roots (Figure 3D). Expression was observed even in lateral roots originating in the hypocotyl (Figure 3E) (these roots are termed 'adventitious roots'). In 17 day old seedlings containing construct B staining in root tissue was still restricted to vascular tissue and very little staining was observed in lateral roots (Figure 3F). In the hypocotyl, expression could be detected in cortical and epidermal cells as well as in vascular tissue (unpublished data). In the upper hypocotyl more extensive staining was apparent in the region near the apical

Fig. 2. Histochemical localization of expression in seeds and seedlings from representative plants. (A) Domain A in seed. (B) Domain A in endosperm. (C) Domain B in seed. (D) Domains A + B in seed. (E) Domain A in 6 day seedling. (F) Domain B in 6 day seedling. (G) Domains A + B in 6 day seedling. (H) Construct 2 (-72 to $+8$) in 6 day seedling. (I) Domain A in root of 10 day seedling. (J) Domain B in root of 10 day seedling. (K) Domains A + B in root of 10 day seedling. (L) Construct 2 (-72 to $+8$) in root of 10 day seedling. Abbreviations: Ra, radicle; Rp, radicle pole of endosperm; C, cotyledon; Cp, cotyledon pole of endosperm; En, endosperm; R, root; S, stele (vascular tissue of hypocotyl); V, vascular tissue; Rc, root cap.

meristem as well as in the cotyledons and young leaves (unpublished data). For plants containing domains A + B expression in vascular tissue as well as in lateral roots was observed (Figure 3G). In addition strong expression in root and in most cells of the hypocotyl and of the cotyledons and leaves was observed. There was no detectable expression in any tissue of seedlings containing construct 2.

We quantitated expression from representative plants for each of the constructs by dissecting the seedlings into upper and lower regions for six day seedlings, and into cotyledons, hypocotyls and roots for 15 day seedlings. The GUS enzyme activities were consistent with the histochemical observations (Table I).

We conclude that in germinating seedlings there are two distinct expression patterns conferred by the two domains (Table II). Domain A confers expression principally in the root tip, in lateral roots, and in a region below the apical meristem. Expression from domain B is highest in the cotyledons and in the vascular tissue of the hypocotyl and root. The expression pattern when both domains are present in the same construct appears to be somewhat more than the sum of the patterns of the domains alone. This is most apparent in the 10 day seedling, particularly in the staining of cortical and epidermal cells of the hypocotyl and root which do not stain with either domain alone. In addition, quantitation of enzyme activity suggests a synergistic interaction between the two domains resulting in higher levels of expression, particularly in root and hypocotyl, when both domains are present (Table I).

Expression at seven weeks

The plants were maintained in tissue culture for seven weeks. Fresh sections were cut from younger and older leaves, from upper and lower stems and from roots. To better visualize the histochemical dye, the sections from leaf and stem were stained, then fixed and placed in ethanol to remove chlorophyll.

Plants that contain domain A showed high expression in the roots. Expression was strongest in the cells at the root tip, particularly in the cortex and in the meristematic region (Figure 3H), but staining was also detected in the epidermis and in root hairs. Staining was notably absent from vascular tissue in the root. Expression was also frequently detected in the layer of cells surrounding the vascular tissue (Figure 3I). This layer of cells includes the pericycle and may include some endoderm cells. Staining of these cells was generally observed only in regions of the root from which lateral roots were forming (Figure 3J). Very light expression was also detected in vascular tissue of younger leaves and in the upper stem (unpublished data). Plants with construct 2 showed no expression in any tissue.

In seven week old plants that contain domain B expression in the root was detected principally in vascular tissue.

Expression restricted to vascular tissue at the site of lateral root formation (Figure 3K) was in contrast to expression in the pericycle layer from domain A (Figure 3J). In the leaf, expression from domain B was detected in mesophyll, vascular and epidermal cells (Figure 3L). In the stem expression was strong in cortex, pith and vascular cells (unpublished data). In plants that contain both fragments A + B, expression in the root was detected in the vascular tissue and the pericycle as well as in most other cell types. In the leaf and stem, expression from fragments A + B was indistinguishable from expression from fragment B alone.

Discussion

Expression conferred by domain A

Expression conferred by domain A (−90 to +8) was particularly strong in root tissue or tissue destined to become root (radicle in the seed) (Table II). Since the deletion of 18 bp results in the loss of detectable expression it would appear that the *cis*-element responsible for this expression is located between −90 and −72. Another possibility is that the *cis* element spans the breakpoint at −72. A *cis*-element located between −90 and −59 has been shown to bind a factor found in extracts of tobacco tissue (Lam *et al.*, 1989). Site specific mutations that inhibit factor binding *in vitro* cause decreased expression in the root in transgenic plants. Addition of this binding site to the promoter from the pea *rbcS3A* gene which is normally expressed only in green tissue resulted in high expression in the root (E.Lam *et al.*, submitted). In the factor binding site there is a tandem repeat of the sequence TGACG separated by 7 bp. Mutation of two bp within the upstream TGACG sequence causes inhibition of binding to this element *in vitro* (E.Lam *et al.*, 1989). Since the upstream TGACG sequence is located between −90 and −72 it is probable that deletion of this sequence also results in inhibition of binding to this region. Therefore it seems likely that the element responsible for the expression pattern observed in seedlings and in plants containing construct A is related to this factor binding site.

Analysis at different developmental stages led to the observation that expression was particularly strong in lateral roots and in the pericycle which is the cell layer from which lateral roots develop. This suggests the possibility that the signal for induction of lateral root formation may activate expression from fragment A. Experiments to investigate this possibility are underway.

Expression conferred by domain B

Expression conferred by domain B (−343 to −90) was strongest in the cotyledons of seeds and seedlings, and in the vascular tissue of the hypocotyl. Lower levels of expression were detectable in most other tissues of the seedling, with lowest levels found in non-vascular tissue of the root. It

Fig. 3. Histochemical localization of expression in seedlings and 7 week old plants from representative plants. (A) Domain A in apical meristematic region of 10 day seedling. (B) Domain B in apical meristematic region of 10 day seedling. (C) Domain B in the cotyledon of 10 day seedling. (D) Domain A in lateral root of 17 day seedling. (E) Domain A in the hypocotyl with newly formed lateral root of 17 day seedling. (F) Domain B in the root with newly formed lateral roots of 17 day seedling. (G) Domains A + B in the hypocotyl and root with newly formed lateral roots of 17 day seedling. (H) Domain A in longitudinal section of root of 7 week old plant. (I) Domain A in transverse section of root of 7 week old plant. (J) Domain A in transverse section of root of 7 week old plant at site of lateral root formation. (K) Domain B in transverse section of root of 7 week old plant at site of lateral root formation. (L) Domain B in leaf of 7 week old plant. Abbreviations as in Figure 2 with following additions: A, apical meristem; Co, root cortex; LR, lateral root; P, pericycle; M, mesophyll; Ep, epidermis.

appears therefore that expression conferred by domain B is detectable in nearly all cell types, but is lowest in those cell types in which expression is highest from domain A. We conclude that domain B is responsible for expression in most cell types other than non-vascular root tissue. It is apparent that this division between expression from domains A and B is not absolute. For example, both domains can confer expression in the vascular tissue of the cotyledon and leaf during certain stages of development.

Since domain B is able to confer expression in many cell types it is possible that this domain is made up of several *cis*-elements, each of which has a greater degree of specificity for expression in a particular cell type or during a particular stage of development. Preliminary experiments indicate that this is the case (P.N.Benfey, in preparation).

Expression conferred by domains A + B

Expression from the construct containing both domains A and B appeared to be higher than in plants containing either domain alone and was detected in additional cell types at certain stages of development. Analysis of expression in mature leaves from deletion derivatives of the 35S promoter indicated that domain A was able to increase expression from a fragment from -343 to -208 which, when fused to a minimal 35s promoter (-46 to +8) showed no detectable expression (Fang *et al.*, 1989). From the GUS enzyme activity assay a similar synergistic interaction appears to result from fusion of domain B to domain A.

Expression in seeds

The expression pattern conferred by the different domains in mature seeds was of particular interest since the expression

of seed storage genes has been localized to specific regions of the seed (for review see Goldberg *et al.*, 1989). Expression from the α' subunit of β conglycinin was localized to the cotyledons and upper axis cells of the embryo by *in situ* hybridization (Barker *et al.*, 1988). The promoter of a wheat glutenin gene conferred expression of a CAT reporter gene to dissected endosperm tissue and not to embryo tissue (Colot *et al.*, 1987). Expression from a maize zein gene promoter fused to the GUS coding sequence was detected by histochemical localization only in endosperm tissue of transgenic tobacco (Scherthner *et al.*, 1988). In contrast, expression from domain A was detected in the radicle of the embryo and expression from domain B was detected primarily in the cotyledons. In addition, each domain conferred a specific pattern of expression in the endosperm. This is of interest since, to our knowledge, no morphological or biochemical difference among endosperm cells of tobacco has been previously reported (see for example, Avery, 1933).

Variation among independent transgenic plants

In this analysis we were interested in studying differences in transcriptional regulation conferred by the two domains. Since the RNA species and protein products produced from the four constructs should be identical, we conclude that the different expression patterns we observed are due to differences in transcriptional activity. However, the use of histochemical localization to detect cell specific expression is not without potential problems. Differences in cell size and metabolic activity, as well as penetration of the substrate into the cell, can contribute to differences in staining intensity (see Jefferson *et al.*, 1987). We attempted to minimize these factors by use of both positive (construct 4) and negative controls (construct 2) and by analysis of at least eight independent transgenic plants for each construct. We did observe variation in the degree of staining among the plants containing constructs 1, 3 and 4 (construct 2 was always without staining in all tissues). For construct 3, 9 of the 10 plants analyzed showed the staining pattern described above, but with varying degrees of intensity in the tissues described. One plant containing construct 3 showed more extensive expression in the root epidermal tissue and root hairs than did the other 9 plants analyzed, however this expression was not similar to that observed from construct 4. There was also one plant containing construct 1 that showed more extensive expression in the cotyledon during seedling development. In this case expression was particularly strong in the root. The possible reasons for variation among independent transgenic plants are several. Differences in copy number of the transgene and in allele number (heterozygote versus homozygote) can contribute to variation. We performed Southern blots on three plants containing fragment A which showed large differences in expression levels. We

Table I. GUS activity in seedlings

10 day seedlings			
Construct domain	Upper		Lower
1 A	880		6600
2 (-72 to +8)	22		22
3 B	11 880		4400
4 A + B	63 800		39 600
15 day seedlings			
Construct domain	Leaf	Stem	Root
1 A	440	2640	24 200
2 (-72 to +8)	44	66	132
3 B	178 200	35 200	17 600
4 A + B	118 800	74 800	220 000

Results from representative plants in pmol MU/mg protein/min.
Results for construct 2 are very close to readings from extracts from untransformed plants.

Table II. Expression patterns conferred by domains of the 35S enhancer

Domain	Seed		Seedling		
	Embryo	Endosperm	6 d	10 d	17 d
A	Radicle	Radicle	Root	Root and apex	Root and apex
B	Cotyledon	Cotyledon	Vascular in root and hypocotyl, cotyledon	Vascular in root and hypocotyl, cotyledon	Vascular in root and hypocotyl, cotyledon leaf
A + B	Radicle cotyledon	Radicle cotyledon	All cells	All cells	All cells

detected, at most, a 2-fold difference in copy number (unpublished data). The most likely cause of quantitative variation in expression is due to different sites of integration in the chromosome (Odell *et al.*, 1985; Sanders *et al.*, 1987). This 'position effect' may be due to insertion near *cis*-elements (positive or negative) that can influence expression from the transgene. Another possibility is that the interaction between *trans*-factors and *cis*-elements of the introduced DNA is influenced by the site of integration.

Since expression from all the constructs (except construct 2) differed with developmental stage, in order to make reproducible comparisons between expression patterns conferred by the promoter fragments, we found that it was essential to analyze expression at defined developmental stages. We note that we observed more extensive expression from the 35S promoter (construct 4) in the stem of 7 week old plants than the phloem specific expression reported previously (Jefferson *et al.*, 1987). This may be due to differences in the construct introduced into plants or to differences in the developmental stage analyzed.

Conclusion

We have characterized the expression conferred by two domains present in the 35S upstream region. The two domains confer different expression patterns in seeds, seedlings and 7 week old plants. Analysis of the simian virus 40 (SV40) large T antigen promoter indicated that its constitutive expression is conferred by multiple *cis*-elements. When these *cis*-elements were isolated and multimerized they conferred different levels of expression in different cell lines (Nomiya *et al.*, 1987; Ondek *et al.*, 1987; Schirm *et al.*, 1987). Our results indicate that the 35S promoter is also constituted of at least two *cis* elements. The use of transgenic plants and histochemical localization has allowed us to define the expression pattern in particular cell types and at different stages of development.

The use of multiple *cis*-elements to confer constitutive expression may be specific to viral promoters which have been selected for the ability to give high level expression in many cell types and under diverse metabolic conditions. It is also possible that normal cellular constitutive promoters (for example, promoters of housekeeping genes) are similarly organized. Characterization of the promoters of constitutive genes (viral or cellular), can, therefore, lead to the identification of multiple *cis*-elements each able to confer a different type of transcriptional regulation. Identification of the *trans*-factors that interact with these elements should help to elucidate the regulatory pathways that determine development in higher plants.

Materials and methods

Constructs

Construct 1 is the same as X-GUS-90 described in Benfey and Chua (1989). Construct 2 was made essentially in the same manner as construct 1 except that a 35S fragment from -72 to +8 was fused to the GUS coding sequence as a *Clal* (5'), *HindIII* (3') fragment. The *HindIII* site was filled in with Klenow enzyme. The *Clal* (5'), *EcoRI* (3') fragment containing the 35S -72 to -8 fragment fused to the GUS coding sequence with a 3' end from the pea *rbcs3C* gene was then inserted between the *Clal* and *EcoRI* sites of the polylinker of pMON505 (Horsch and Klee, 1986). A construct containing the 35S promoter (-941 to +8) fused to the chloramphenicol acetyl transferase (CAT) coding sequence with a 3' end from the pea *rbcsE9* gene was inserted into the *HpaI* site 4 kb away from the GUS construct.

CAT activity was measured to confirm that all plants were transformed. Construct 3 was made by inserting a fragment from the 35S promoter deleted to -343 with attachment of a *HindIII* linker (as described in Odell *et al.*, 1985) and cut at the *EcoRV* site at -90 with attachment of a linker that contained an *XhoI* site, between the *HindIII* and *XhoI* sites upstream of the *Clal* site in construct 2. Construct 4 was made by inserting the same 35S fragment from -343 to -90 between the *HindIII* and *XhoI* sites of construct 1.

Transgenic plants

The constructs were mobilized into a 'disarmed' *Agrobacterium tumefaciens* strain GV3111SE by triparental mating (Rogers *et al.*, 1986). Exconjugants were used to inoculate leaf disks of *Nicotiana tabacum* cv. SR1 and regenerated shoots were selected on medium containing kanamycin (200 µg/ml) (Rogers *et al.*, 1986). After rooting, transgenic plantlets were transferred to soil and grown in a greenhouse. The primary transformants were allowed to self-fertilize and seeds were collected. For the studies on expression, seeds were sterilized and germinated on a media containing MS salts, 3% sucrose, 0.7% agar, 100 µg/ml kanamycin, and 500 µg/ml carbenicillin. The seedlings were maintained at 26°C in a cycle of 16 h light, 8 h dark. After approximately 21 days, 2 seedlings from each transgenic plant were transferred to a Plantcon (Tm) containing the same media where they continued to grow under the same environmental conditions.

Histochemical staining

Histochemical staining was performed as described (Jefferson, 1987) with the following modifications. Mature seeds were deposited in a dense monolayer in cyanoacrylate adhesive (Krazy Glue TM) placed on a section from a carrot. The carrot section was attached to the block used for sectioning supplied with the Vibrotome (TM) sectioning device. Sections of 100 to 200 microns were cut with the Vibrotome and placed directly in the histochemical substrate solution of 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) and 50 mM sodium phosphate buffer (pH 7.0) on a microscope slide on which a thin beading of Vaseline had been placed around the edge. For some sections the embryos were manually removed from the endosperm with a dissecting needle prior to incubation. The sections were incubated for 12 to 16 h in a humidified chamber at 37°C. Coverslips were placed on the slides before viewing.

Six day old seedlings were removed from Petri dishes, placed directly in the X-gluc solution and incubated as described above for the seeds. Ten and seventeen day old seedlings were removed from Petri dishes and placed in a small amount of X-gluc solution on a microscope slide. The seedlings were then pressed with a second microscope slide. The pressed seedlings were then removed to a fresh microscope slide with X-gluc solution and incubated as described above for seeds.

For seven week old plants, fresh sections were hand cut. Sections from root were placed directly in X-gluc solution and incubated as described above. Sections from stem and leaf were incubated with the X-gluc solution in 24-well microtiter dishes for 12–16 h at 37°C, then cleared of chlorophyll by incubation for 10 minutes in a solution of 5% formaldehyde, 5% acetic acid, and 20% ethanol, followed by incubation for 2 min in 50% ethanol, 2 min in 100% ethanol, and two washings in distilled water. The sections were then mounted on microscope slides for photography. Photomicrographs were taken with a Nikon Optiphot microscope using phase contrast optics.

GUS enzyme assays

GUS enzyme assays were performed essentially as described (Jefferson *et al.*, 1987). Extracts were made from upper and lower portions of six day old seedlings that were cut in the middle of the hypocotyl, and from 15 day old seedlings that were dissected into roots, hypocotyl and cotyledons (and young leaves). Five µg of protein were incubated with 4-methyl umbelliferyl glucuronide (MUG) solution for 15 minutes after which 2.5 ml of 0.2 M sodium carbonate were added. Fluorescence was measured with a Perkin-Elmer LS5 fluorimeter as described (Jefferson *et al.*, 1987). Fluorescence of a solution of 100 nM 4-methyl umbelliferone (MU) in 0.2 M sodium carbonate was used for calibration.

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