Regulated Genes in Transgenic Plants

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Transgenic plants are an effective system for the study of regulated gene expression. Developmental control of expression can be monitored by assaying different tissues or by assaying a plant at different developmental stages. Analysis of the petunia 5-enolpyruvylshikimate-3-phosphate synthase gene, which is highly expressed in flowers, allowed identification of an upstream region that confers tissue-specific and developmentally regulated expression. The cell specificity of expression in floral tissues has been defined by histochemical localization. This expression is contrasted to that of the 35S promoter of cauliflower mosaic virus, a nominally constitutive promoter that shows a definite specificity of expression in floral tissues. Moreover, this expression differs in transgenic hosts of different species.

THE ABILITY TO ANALYZE THE EXPRESSION OF A MODIFIED gene in a multicellular organism has contributed greatly to the understanding of gene regulation. Transgenic organisms are of particular use in the study of development, since gene expression can be analyzed at different stages and in various tissues.

In higher plants, DNA can be introduced into somatic cells by *Agrobacterium*-mediated (*1*) or direct gene transfer (*2*); the transferred DNA is referred to as a transgene. Plants that contain the transgene in all cells can then be regenerated and can transfer the transgene to their offspring in a Mendelian fashion. The ease of producing transgenic plants has permitted the use of this system for the analysis of gene expression in various developmental and environmental conditions. Recent studies of light responsiveness that varies with developmental stage and studies of expression restricted to a particular tissue or developmental stage of an organ have begun to exploit the potential of this system.

In this review, we concentrate on recent studies of transgenic plants not covered in other reviews (*3–5*). In addition, we report on the developmental and tissue-specific regulation of 5-enolpyruvylshikimate-3-phosphate synthase, which is expressed at high levels in flowers. We map sequences responsible for this regulated expression to an upstream region of the promoter. We use histochemical localization to define the cell specificity of expression within the floral tissues and compare this expression to that of a nominally constitutive promoter that shows a high level of cell specificity in floral tissues. Differences in expression patterns for both genes in two closely related transgenic hosts are described.

Inducible Genes

The environmental stimuli influencing gene expression that have been investigated with transgenic plants include light, heat, anaerobic stress, and wounding. Regulation of gene expression by an internal circadian clock has also been shown (Table 1). Although the use of plant cell lines, protoplasts, and undifferentiated callus tissue has yielded important results in the understanding of inducible genes (*6–8*) we limit our discussion in this review to work in transgenic plants.

Light is perceived by the plant through photoreceptors that have characteristic absorbance spectra. Phytochrome, the best characterized photoreceptor, interconverts between two forms: P_{fr} when red light is absorbed and P_r when far-red light is absorbed (*9*). Initial experiments [reviewed in (*4, 5*)] demonstrated that the light and phytochrome responsiveness of two gene families involved in photosynthesis, those encoding the small subunit of ribulose bisphosphate carboxylase (*rbc*S) and the chlorophyll a/b binding protein (*Cab*), could be reproduced when either whole genes or upstream regions of the genes driving heterologous coding sequences were introduced into transgenic plants (*10–13*).

Further analysis of expression from these genes has led to the identification of the sequences responsible for light responsiveness,

Table 1. Inducible genes. Abbreviations: *N. plumba*, *Nicotiana plumbaginifolia*; *A. majus*, *Antirrhinum majus*.

Gene	Plant	Transgenic host	Inducer	Reference
*rbc*S	Pea	Tobacco	Light	(*4, 10, 11*)
*rbc*S	Pea	Petunia	Light	(*61*)
*rbc*S	*N. plumba*	*N. plumba*, petunia, tobacco	Light	(*55*)
*rbc*S	Soybean	Petunia	Light	(*62*)
Cab	Pea	Tobacco	Light	(*17*)
Cab	Wheat	Tobacco	Light	(*13, 18*)
Cab	*N. plumba*	Tobacco	Light	(*19*)
Chalcone synthase	*A. majus*	Tobacco	Light	(*54*)
ST-LS1	Potato	Tobacco	Light	(*63, 64*)
Hse	Maize	Petunia	Heat	(*23*)
Hse	Soybean	Tobacco	Heat	(*24, 25*)
ADH	Maize	Tobacco	Anaerobic stress	(*26*)
Protease inhibitor	Potato	Tobacco	Wounding	(*65, 66*)
Cab	Wheat	Tobacco	Circadian clock	(*27*)

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has revealed complex interactions between enhancer elements and promoters, and has shown that redundant elements can play a role in developmentally regulated expression.

Deletion analysis of the pea *rbc*S-3A gene revealed that expression in the light, which remained high with 5′ deletions to nucleotide −166, was greatly reduced when the region between positions −166 and −149 was removed (*14*). With the 5′ boundary of a positive element thus defined, mutations were made in three internal regions chosen because of conserved homology among *rbc*S genes. Disruption of two conserved regions of 12 base pairs (box II) (*15*) or 8 bp (box III) (*15*) caused drastic decreases in expression, but replacement of a third region of 8 bp (box I) had no effect (*16*). Precise localization of the sequences involved in positive regulation was further specified when 2 bp within box II were mutated from GG to CC and expression was reduced to undetectable levels (*16*). Less precisely defined are the elements required for expression from the *Cab* genes. A 247-bp region (−347 to −100) from the pea *Cab AB80* gene was able to confer light responsiveness in an orientation-independent manner on a heterologous TATA sequence fused to the neomycin phosphotransferase [NPT (II)] coding sequence (*17*). A 268-bp fragment (−357 to −89) from the wheat *Cab-1* gene had similar enhancer-like properties (*18*). This fragment could confer inducibility by red light that could be reversed by a flash of far-red light—the hallmark of phytochrome-mediated induction (*18*).

Analysis of the *Cab-E* gene from *Nicotiana plumbaginifolia* indicated the presence of negative elements as well as a complex interaction between light-responsive elements and promoter regions (*19*). Deletion analysis suggested the presence of one negative and two positive regulatory regions. Internal deletions indicated that a fourth region located between −368 and −234 has an essential positive role in light induction. A 210-bp fragment from −396 to −186 was then shown to confer light inducibility on a heterologous promoter. However, when a larger region of the *Cab-E* gene (−1554 to −112) (which contained the sequences of the entire smaller fragment) was fused to the same promoter, equal levels of expression were observed in the light and the dark. When the same region was fused to its own promoter, it again conferred light responsiveness (*19*). Negative regulatory effects have also been observed for the box II region of the *rbc*S-3A gene. This region was multimerized and inserted between the TATA box and the upstream region of the cauliflower mosaic virus (CaMV) 35*S* promoter (*14*). Expression became light-responsive, apparently as a result of attenuation of expression in the dark from the normally constitutive 35*S* promoter (*14*).

Redundancy in light-responsive elements was detected when the mutations in the *rbc*S-3A gene that reduce expression of the promoter with a 5′ boundary at nucleotide −170 were shown to have no effect when the promoter fragment was extended to position −410 (*16*). The functional redundancy of positive elements was confirmed when the region between −410 and −170 was shown to be sufficient to confer light responsiveness when placed upstream from a heterologous TATA region (*16*). Additional redundancy may exist. Work on callus tissue indicated that the *rbc*S TATA region is involved in light regulation (*7*). Experiments on transgenic tobacco in which heat shock elements were used upstream from the *rbc*S-3A TATA region (−50 to +15) indicate that this region by itself can confer light-responsive expression (*20*).

The potential of the transgenic plant system was more fully explored when leaves at different developmental stages and harboring two deletion derivatives of the *rbc*S-3A upstream region were analyzed. No difference in expression was detected in mature leaves between a fragment with a 5′ boundary at −410 and a shorter fragment with a 5′ boundary at −166. However, in young developing leaves, expression from the shorter fragment was greatly reduced (*16*). In addition, expression from the shorter promoter was lower in etiolated seedlings exposed to white light for 24 hours (*16*). The region from −410 to −166, therefore, appears to be essential for high level expression in immature tissue. The ability to examine different developmental stages of an organ from the same plant was critical in defining a new role for apparently redundant sequences.

Thermal stress produces rapid changes in gene expression, inducing the transcription of a small set of heat shock genes and reducing the RNA levels of many other genes (*21*). The high degree of conservation of this response among evolutionarily diverse organisms was demonstrated when the upstream region of the *Drosophila* heat shock gene *hsp70* was fused to the NPT (II) coding region and introduced into tobacco. Heat-inducible expression of the encoded enzyme showed the same organ specificity (inducibility in all organs except pollen) as was shown by endogenous plant heat shock genes (*22*). The maize homolog of *hsp70* with 1.1 kilobases of upstream sequence also showed heat inducibility in transgenic petunia (*23*). Deletion analysis of a soybean heat shock gene, *hsp6871*, indicated that both consensus heat shock elements and an enhancer element were necessary for optimal heat inducibility of transcription in tobacco (*24*). A 36-bp region (which contains two heat shock elements) from the same gene was placed in front of a 35*S* TATA region. This was sufficient to confer heat inducibility of the RNA in transgenic tobacco. Induction was not dependent on light and was detectable in all organs tested (*25*). However, when this 36-bp element was placed upstream from the *rbc*S-3A promoter, expression under heat shock conditions was at high levels only in leaves and only in the light (*25*). One explanation for these results is that negative elements present in the *rbc*S-3A promoter suppress expression under heat shock conditions in the dark and in organs (roots) in which *rbc*S-3A is not normally expressed. In these transgenic plants a novel pattern of expression was generated—a light-dependent and organ-specific heat shock response.

The changes in gene expression that accompany oxygen deprivation in maize seedlings are not unlike those of the heat shock response. Among the small set of induced genes is the one that codes for alcohol dehydrogenase (*ADH*). When the upstream region of the maize *ADH* gene was fused to a chloramphenicol acetyltransferase (CAT) coding sequence and introduced into tobacco, only background CAT activity was detectable under aerobic or anaerobic conditions (*26*). However, insertion of enhancer-like regions from the constitutive octopine synthase (*OCS*) or CaMV 35*S* gene upstream from a 247-bp fragment of the *ADH* gene resulted in clear induction under anaerobic conditions (*26*). These results can be interpreted as indicating that downregulation during aerobic conditions is conferred by the *ADH* gene sequences.

Initial observations that transgenic plants containing the wheat *Cab-1* gene show higher mRNA levels after reillumination than before dark treatment (*12*) led to a closer study of circadian rhythms. The entire *Cab-1* gene and a second construct containing the upstream region of the *Cab-1* gene fused to the CAT coding sequence were introduced into tobacco. Cycling of mRNA levels with a 24-hour periodicity was observed from both constructs (*27*). Cycling was maintained under both continuous dark and continuous light conditions, an indication of control by an endogenous circadian clock (*27*). A third construct, which contained a truncated *Cab-1* promoter region, the *Cab-1* structural gene, and a 35*S* enhancer at the 3′ end, showed constant levels of expression throughout the 24-hour period. This indicated that control by the circadian clock was at the transcriptional level, since the *Cab* mRNA transcribed from this construct was identical to the mRNA transcribed from the construct that exhibited cycling (*27*).

From work on inducible expression in transgenic plants a few conclusions can be drawn. Redundant elements have been identified

in two genes (*rbc*S-3A and the soybean heat shock gene *hs6871*). Whether functional redundancy is a general feature of plant promoters is difficult to ascertain, since most promoter mapping has involved deletions that tend to mask the presence of redundant elements. Redundancy can be detected through "gain of function" experiments in which small DNA fragments are inserted upstream from a heterologous TATA region. However, choice of the TATA region must be made judiciously in view of the complex interactions demonstrated with the *Cab*-E upstream region and TATA region and the fact that the *rbc*S-3A TATA region plays a role in light responsiveness. Transgenic plants are particularly well suited to the analysis of gene regulation that differs with developmental stage, as shown for the *rbc*S-3A promoter; they are also useful for the analysis of expression that is tissue specific.

Tissue-Specific Genes

Faithful reproduction of endogenous expression patterns has been found for transgenes expressed in green tissue, tubers, nodules, seeds, and now, flowers (Table 2). The light-inducible genes discussed above are expressed in chlorophyll-containing tissue. In transgenic tobacco, the pea *rbc*S-3A gene (*4*), the pea *rbc*S *ss3.6* gene (*28*), the wheat *Cab-1* gene (*12*), and the pea *Cab AB80* gene (*11*) showed high expression in leaves, lower expression in stems, and undetectable levels in roots. Activity of NPT (II) was assayed in dissected epidermis, mesophyll, and midrib from leaves of transgenic plants with constructs containing the upstream region of the *Cab AB80* gene or the *rbc*S *ss3.6* gene fused to the NPT (II) coding region (*28*). For both genes activity was detectable only in the mesophyll tissue, which has the highest concentration of chlorophyll-containing cells. A more detailed analysis was performed with indirect immunolocalization of CAT protein expressed from an *rbc*S 3A (−410 to −50) sequence upstream from a CaMV 35*S* TATA region fused to the CAT coding sequence. In transgenic tobacco CAT protein was localized to the chlorophyll-containing cells, including guard cells in the epidermis, chlorenchyma, and vascular cells of the midrib and mesophyll cells in the lamina (*29*).

In all of the photosynthetic genes mentioned above, the region responsible for light responsiveness is identical to that conferring tissue specificity. Both the upstream (−410 to −170) and downstream (−166 to end of gene) regions of the *rbc*S 3A gene that confer light responsiveness also confer green tissue-specific expression (*16*). This finding illustrates again the apparent reiteration of genetic information. In one case, however, a region able to provide expression in green tissue was separated from sequences conferring light responsiveness. Initial analysis of the *Nicotiana plumbaginifolia rbc*S 8B gene in transgenic *N. plumbaginifolia* revealed a pattern similar to those of the pea *rbc*S genes; sequences between −312 and −102 could confer light inducibility and green tissue specificity on a heterologous promoter (*30*). Sequences farther upstream, however, behaved differently. A fragment from −1038 to −93 could confer light responsiveness on a CaMV promoter fragment fused to the CAT coding sequence. But the upstream region from −1038 to −589 fused to the same promoter showed equal levels of RNA in the light and dark. The CaMV promoter fragment (−105 to +8) used in these studies showed low-level root-specific expression. Addition of the longer *rbc*S 8B region suppressed root expression and increased leaf and stem CAT enzyme levels. In contrast, the shorter fragment caused no change in root expression but increased expression in leaves (*30*). Thus, the shorter region, which does not confer light responsiveness, can enhance expression in leaf tissue.

The 5′ region of a light-responsive gene from potato, *ST-LS1*, was used to express a tuber-specific gene, patatin, in the leaves and stems of tobacco (*31*). Conversely, the 5′ region of the patatin gene when fused to the CAT coding sequence directed tuber-specific expression of CAT enzyme activity in transgenic potatoes (*32*). These results confirm the primacy of 5′ sequences in determining tissue-specific expression in transgenic plants.

The temporal and cell-specific expression of genes for seed storage proteins are tightly regulated. Genomic fragments containing the French bean β-phaseolin gene (*33*), the soybean β-conglycinin α′-subunit (*34*) or β-subunit gene (*35*), the soybean lectin gene (*36*), two alleles of the bean phytohemagglutinin gene (*37*), and the pea legumin gene (*38*) have been transferred to plants and shown to be expressed specifically in seeds. The timing of the onset of expression has generally reflected that of the gene in its endogenous setting. This is true even in cases where the anatomy or the duration of seed development is quite different in the source plant (*33–37*).

Information about the sequences required for seed-specific regulation came from two experiments involving entire genomic regions. A 17.1-kb genomic fragment that contained the soybean lectin *L1* gene and at least four other non-seed protein genes was introduced into tobacco. The non-seed protein genes all showed constitutive expression, whereas the lectin gene was expressed only in seeds and roots (but to a much lower extent in the latter) (*36*). This experiment demonstrated that correct expression of several genes on a large genomic fragment was retained in a transgenic plant. In the second experiment two alleles of a bean lectin gene (*PHA-L*), which show marked differences in expression levels in different bean cultivars, maintained these differences in transgenic tobacco (*37*). Comparison of the upstream sequences suggested that an apparent deletion in one allele may be responsible for its reduced expression (*37*). Flanking sequences of the bean β-phaseolin gene (*39*) or the barley B1 hordein gene (*40*) were sufficient to give seed-specific expression of a maize zein coding region or the CAT coding sequence, respectively.

Sequences responsible for the specificity of expression of two genes have been mapped. In the upstream region of the gene for the α′-subunit of soybean β-conglycinin, seed specificity was main-

Table 2. Tissue-specific genes.

Gene	Plant	Transgenic host	Organ/ tissue	Reference
*rbc*S	Pea	Tobacco	Green	(*4, 10, 11*)
*rbc*S	Pea	Petunia	Green	(*61*)
*rbc*S	*N. plumba*	*N. plumba*, petunia, tobacco	Green	(*55*)
Cab	Pea	Tobacco	Green	(*28*)
Cab	Wheat	Tobacco	Green	(*12*)
ST-LS1	Potato	Tobacco	Green	(*63, 64*)
Patatin	Potato	Potato	Tuber	(*32*)
Leghaemoglobin	Soybean	Lotus	Nodule	(*67–69*)
Nodulin N23	Soybean	Lotus, *Trifolium*	Nodule	(*70*)
β-Phaseolin	French bean	Tobacco	Seed	(*33*)
β-Conglycin α′-subunit	Soybean	Petunia	Seed	(*34*)
		Tobacco	Seed	(*41, 42*)
β-subunit	Soybean	Tobacco	Seed	(*35, 44*)
Lectin	Soybean	Tobacco	Seed + root	(*36*)
Lectin	Bean	Tobacco	Seed	(*37*)
Glutenins	Wheat	Tobacco	Seed	(*43*)
Hordein	Barley	Tobacco	Seed	(*40*)
Zein	Maize	Tobacco	Seed	(*47*)
Legumin	Pea	*N. plumba*	Seed	(*38*)
EPSP synthase	Petunia	Petunia	Flower	This work
		Tobacco	Pollen	This work

tained with 159 bp 5′ of the transcriptional start site, but at a very low level. Expression was significantly increased with an additional 49 bp of upstream sequence, thereby indicating the possible presence of two elements—one defining cell specificity and the other providing positive enhancement (*41*). An alternative explanation is the presence of redundant genetic elements as observed for the *rbc*S-3A gene. The region containing these two putative elements was introduced at three positions in a construct containing the full promoter of the 35*S* gene, the CAT coding region, and the 3′ tail of the α′-subunit gene (*42*). When inserted at position −90 of the 35*S* promoter in either orientation, 25 times greater expression than that of the construct without the insert was observed at the expected stage of seed development. At the 3′ end of the CAT coding sequence, two- to fivefold enhancement was observed, and at the 3′ end of the polyadenylate [poly(A)] addition site there was not apparent enhancement (*42*). This was evidence that this region contained an element that could specifically regulate expression in certain stages of seed development. Whether this element can confer regulated expression when fused to a heterologous minimal TATA box region is not known. Deletion analysis of a wheat glutenin upstream sequence fused to the CAT gene in transgenic tobacco showed that as little as 326 bp but more than 160 bp were necessary for seed-specific expression (*43*).

Localization of expression at the cellular level as opposed to the organ level is desirable for a better understanding of the controls that cause adjacent cells to differentiate into morphologically different cell types. In the studies of seed-specific expression, dissection into embryo and endosperm has shown preferential expression of the protein in one tissue type (*33, 34, 36, 37*). The level of regulation at the cell level is unclear, however, since translational or post-translational processes can and, at least in one instance (*39*), clearly do influence accumulation of the seed storage proteins in transgenic plants. One way to determine cell specificity and focus on regulation at the RNA level is the use of in situ hybridization. β-Conglycinin mRNA was shown by this method to accumulate only in the cotyledons and upper axis cells of the developing embryo (*44*). An alternative method is the use of reporter genes, which has the advantage that, through use of the proper controls, transcription rather than steady-state RNA levels can be monitored. Assays of dissected seeds containing the wheat glutenin gene fused to the CAT coding sequence indicated that most of the CAT activity was present in the endosperm, a result that is consistent with the cell-specific expression of the glutenin gene in wheat (*43*). That this was transcriptional and not translational regulation was shown by assaying a 35*S*-CAT construct, which gave equal levels in both tissues (*43*). Similar results were found for the barley B hordein–CAT construct, CAT enzyme activity being found only in the endosperm; a CaMV 19*S*–CAT construct gave expression in both tissues (*40*). A recently developed reporter gene system that makes use of the *Escherichia coli* β-glucuronidase (GUS) coding region (*45, 46*) was used to detect weak but highly specific expression of a maize zein promoter in transgenic tobacco (*47*). The advantage of this system is the availability of a substrate for use in histochemical staining and the virtual absence of background staining in most tissues (*46*). With the zein promoter, staining was seen in the endosperm but not the embryo of transgenic seeds, whereas a 35*S*-GUS construct showed staining in both tissues (*47*).

This work shows that transgenic plants can be routinely used to identify sequences responsible for tissue-specific and developmentally regulated expression. The trans-acting factors that interact with these sequences are now being characterized (*48*). Once the genes for these factors are cloned, the ability to reintroduce them into transgenic plants should greatly aid in the dissection of regulatory pathways active during development.

Expression of the EPSP Synthase Gene in Transgenic Flowers

Flower development, like seed development, requires the elaboration of specific tissues with specialized functions. To elucidate the genetic controls governing development and differentiation in this complex organ, we examined expression in transgenic plants controlled by sequences from a gene expressed at high levels in flowers.

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase catalyzes a step in the biosynthesis of aromatic amino acids and is the target for the broad-spectrum herbicide glyphosate (*49*). RNA blot analysis of the endogenous gene in mature petunia revealed that RNA levels were very high in petals and barely detectable in leaves (*49*). This was unexpected in view of the apparently essential function of an amino acid biosynthesis enzyme. One potential explanation for the high levels in petals is that phenylalanine is the substrate for anthocyanin biosynthesis which results in the colored pigments found in flowers (*49*). In tomato, however, endogenous EPSP synthase RNA levels were high not only in flowers but also in leaves (*49*).

We examined the tissue specificity conferred by the region upstream from the EPSP synthase gene in the homologous host plant, petunia, and in tobacco, a closely related member of the Solanaceous family. A series of deletion mutants of the region upstream from the petunia EPSP synthase gene, with 3′ end points ranging from −30 [the site of the TATA box (*49*)] to −800, and 5′ end points at approximately −1800, were placed in front of a minimal 35*S* TATA region (−46 to +8), which was fused to the CAT coding sequence in the intermediate vector pMON505-67 (*50*). Analysis of CAT enzyme activity in transgenic petunia showed high activity in petals and near background activity in leaves for all four deletion constructs (Fig. 1A). Expression from the 35*S* TATA

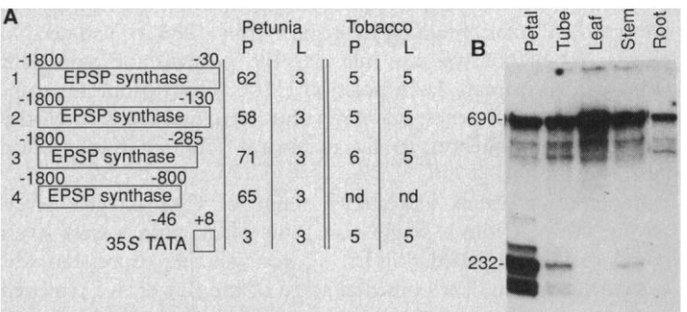

Fig. 1. Deletion analysis of the petunia EPSP synthase promoter. (**A**) Deletions of the EPSP synthase gene promoter were made in a fragment derived from pMON 9561 which contains the 5′ portion of the petunia EPSP synthase genomic clone (*49*). The fragments were subcloned into pMON 505-67 (*50*). The intermediate vector was mobilized into a "disarmed" *Agrobacterium tumefaciens* GV3111SE by triparental mating (*71*). Exconjugants were used to inoculate leaf disks of *Nicotiana tabacum* cv. SR1 or *Petunia hybrida* cv. Mitchell diploid, and regenerated shoots were selected on a medium containing kanamycin (200 μg/ml) (*71*). After rooting, transgenic plantlets were transferred to soil and grown in a greenhouse. Chloramphenicol acetyl transferase (CAT) activity was assayed by the two-phase method (*72*). Five micrograms of protein extracted from mature petals (P) or leaves (L) were incubated for 2 hours at room temperature. Results are for representative plants containing each construct in transgenic petunia or tobacco and are given as counts per minute times 10^{-3}. The number of transgenic plants assayed for each construct in petunia was: 1, 3; 2, 7; 3, 12; 4, 10; 35*S* TATA, 7; in tobacco: 1, 7; 2, 2; 3, 4; 35*S* TATA, 10; nd, not done. (**B**) Organ-specific expression of the −1800 to −285 EPSP synthase promoter region (fragment 3) fused to the 35*S* TATA–CAT construct in transgenic petunia. Total RNA was extracted as described (*18*) from mature petals, floral tubes, leaves, stems, and roots. Twenty micrograms of total RNA were used in quantitative 3′ S1 mapping with a 690-nucleotide end-labeled probe that protects a 232-nucleotide region of the *rbc*S E9 3′ end (*18*). Hybridization and S1 digestion conditions were as described (*18*).

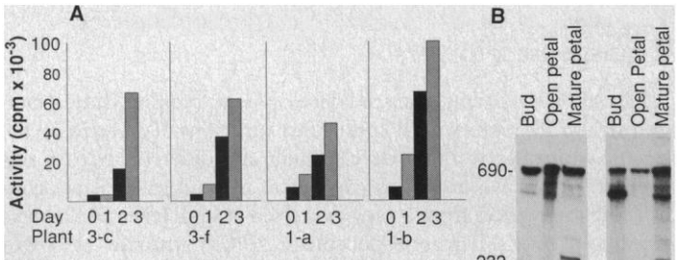

Fig. 2. Developmental regulation of the chimeric EPSP synthase–35*S* constructs in transgenic petunia. (**A**) A portion of the petal harvested 1 day before opening of the flower (day 0) and from the same flower on three successive days (days 1 to 3). Protein was extracted and 5 μg was assayed for CAT activity as described in Fig. 1. Activity for successive days is presented for individual flowers from four independent transgenic plants. Construct 3 contains the EPSP synthase promoter fragment from −1800 to −285; construct 1 contains the fragment from −1800 to −30. (**B**) Total RNA was extracted from petals of three or four flowers 1 day before opening (Bud), 1 day after opening (Open petal), and open for at least 2 days (Mature petal). In (a) the flowers are from construct 1; in (b) the flowers are from construct 3. Twenty micrograms of total RNA were analyzed by quantitative S1 mapping with the *rbc*S E9 probe, as described in Fig. 1B.

region alone in petals and leaves was equivalent to background levels (Fig. 1A). S1 analysis confirmed these observations (Fig. 1B). CAT mRNA from the chimeric EPSP synthase 35*S*-CAT construct 3 was undetectable in the root and leaf; low levels were observed in the stem and floral tube, and high levels were detected in the petals. We conclude that sequences sufficient to confer tissue-specific regulation are located in the EPSP synthase promoter, and at least one copy of these sequences is located upstream from position −800. Assays of nine transgenic plants that contain the promoter fragment from −800 to −285 (two in the forward orientation; seven in the reverse orientation) showed background levels of expression in flowers and leaves (*51*). Plants containing the region from −285 to −30 need to be assayed before we can rule out the presence of redundant elements downstream from position −800. From promoter mapping of other plant genes, it appears unusual to find tissue specificity conferred by sequences so far upstream from the transcription initiation site.

Initial observations indicated a range of CAT enzyme levels among flowers from a single transgenic plant, with lowest levels found in the unopened flowers. To test whether expression was dependent on the developmental stage of the flower, we removed portions of petals on four successive days, starting one day before the flower opened. Four independent transgenic plants containing two different deletion constructs gave similar results (Fig. 2A). CAT activity was low before the flower opened and generally on the first day after it opened, but increased rapidly on subsequent days. In order to rule out wounding during harvesting as the cause of induction of CAT activity, we harvested petals and pooled them from flowers of two independent transgenic plants at three stages each: one day before opening, just after opening, and when flowers were mature (Fig. 2B). A similar increase in CAT activity between flowers that had just opened and mature flowers was evident. The precise timing of the onset of high expression seems to vary somewhat among transgenic plants (Fig. 2A, plants 3-c and 1-b on day 1 after opening), and even among flowers on the same plant (*51*). Changes that are concomitant with cell expansion during petal opening, such as fluctuations in endogenous hormone levels, may be involved in the massive induction of the EPSP synthase promoter.

In contrast to the results from the transgenic petunia, in transgenic tobacco no CAT expression above background could be detected in any organ for any of the constructs tested (Fig. 1A). Petals of flowers at various developmental stages also failed to show any expression above background.

Cell-Specific Expression of the EPSP Synthase Gene Construct

To examine the cell specificity of expression conferred by the EPSP synthase gene sequences in petunia, and with the hope of detecting specific but low level expression in tobacco, we used the GUS reporter gene. The EPSP synthase gene 5′ region (−1800 to −285) was placed upstream from a 35*S* promoter region (−90 to +8) [which alone gives low level expression only in root (*30, 51*)], and this construct was fused to the GUS coding sequence (*52*).

Histochemical localization was used on fresh sections from various floral tissues. Under the conditions used, wild-type tobacco showed no staining in any floral tissue and wild-type petunia showed light staining in the stigma and in anthers and pollen of immature flowers (*51*). With the chimeric EPSP synthase 35*S*-GUS construct in transgenic petunia, intense staining was observed in upper and lower epidermis, vascular tissue, and mesoderm of the mature petal (Fig. 3A). The chlorophyll-containing cells in the midrib of the petal, however, were unstained (Fig. 3B). From serial sections there appears to be a gradient of expression in the floral tube. At the top of the tube the upper epidermis and adjacent cell layers stain (*51*); somewhat farther down in the tube, staining is observed principally in the upper epidermis (Fig. 3C) whereas in the lower tube no staining is detectable (Fig. 3D). Expression limited to specific cells in the tube provided an explanation for the small amount of CAT RNA found in the tube as compared to the petals (Fig. 1B). Also correlating well with the CAT RNA expression data, little staining was detected in sepals (Fig. 3E), a green leaf-like part of the flower (Fig. 3L).

Staining of floral tissues in tobacco revealed the sensitivity of the GUS histochemical assay. While no staining was apparent in most petal sections (Fig. 3F), light staining of epidermal tissue was occasionally observed (*51*). The only floral tissue in tobacco that stained consistently was pollen; the blue staining of approximately 50% of the pollen is suggestive of Mendelian segregation of a single transgene locus (Fig. 3G). This finding prompted us to check the staining in the reproductive parts of the transgenic petunia flowers. We observed fairly uniform staining of immature ovules in which most of the tissue is maternal and diploid (Fig. 3H). However, in the developing seeds, where maternal tissue is negligible, we detected differential staining in adjacent seeds (Fig. 3I). Initial observations revealed that staining in these self-fertilized seeds follows a roughly 3:1 segregation pattern (stained versus unstained), which would be expected of expression from a single chromosomal locus. In pollen of mature flowers we also detected differential staining (Fig. 3J). As in transgenic tobacco, approximately 50% of the pollen was stained, indicating 1:1 segregation of the transgene locus. In wild-type petunia no staining of pollen was observed in mature flowers (*51*).

The developmental activation of expression of the EPSP synthase–35*S* chimeric gene in mature flowers was also reflected in the histochemical staining. Petals from flowers just after opening show light staining (Fig. 3K) as compared to petals from mature flowers (Fig. 3A). The histochemical data indicate that the developmental regulation involves augmented expression in all cells of the petal (except those of the midrib) during the period of cell expansion after opening of the flower. Ten independent transgenic plants of petunia containing the EPSP synthase–35*S* construct showed similar staining patterns, including staining in petals, seeds, and pollen and differential expression between petals and sepals. We did observe

variation in the intensity of staining in the petal; plants in which staining was less intense (five plants) showed little staining in the tube. In tobacco, eight independent transgenic plants were assayed, of which five showed staining of pollen.

From these data, we conclude that sequences between −1800 and −800 of the petunia EPSP synthase gene are sufficient to give high expression in certain floral tissues in the homologous transgenic plant. Expression is clearly dependent on the developmental stage of the flower, a previously unreported aspect of EPSP synthase gene regulation. Preliminary results indicate that the endogenous petunia EPSP synthase gene shows a similar pattern of developmentally regulated expression (*51*). The developmental timing of expression of two petunia anthocyanin biosynthesis genes, encoding chalcone synthase and chalcone isomerase, has been reported (*53*). Expression peaks before the flower opens and decreases in the open petal (*53*). Since EPSP synthase is activated after the anthocyanin genes, it seems unlikely that the high level of expression of EPSP synthase in petals occurs solely to provide substrate for the anthocyanin biosynthesis enzymes.

The cell specificity of expression conferred by the upstream region of the EPSP synthase gene as defined by histochemical staining includes upper and lower epidermis, mesoderm and vascular tissue in the petal, upper epidermis and adjacent cells in the tube, maternal tissue of the ovules, and segregating phenotypes in immature seeds and pollen. It has been reported that the 35*S* promoter region from position −90 to +8 can interact synergistically with upstream

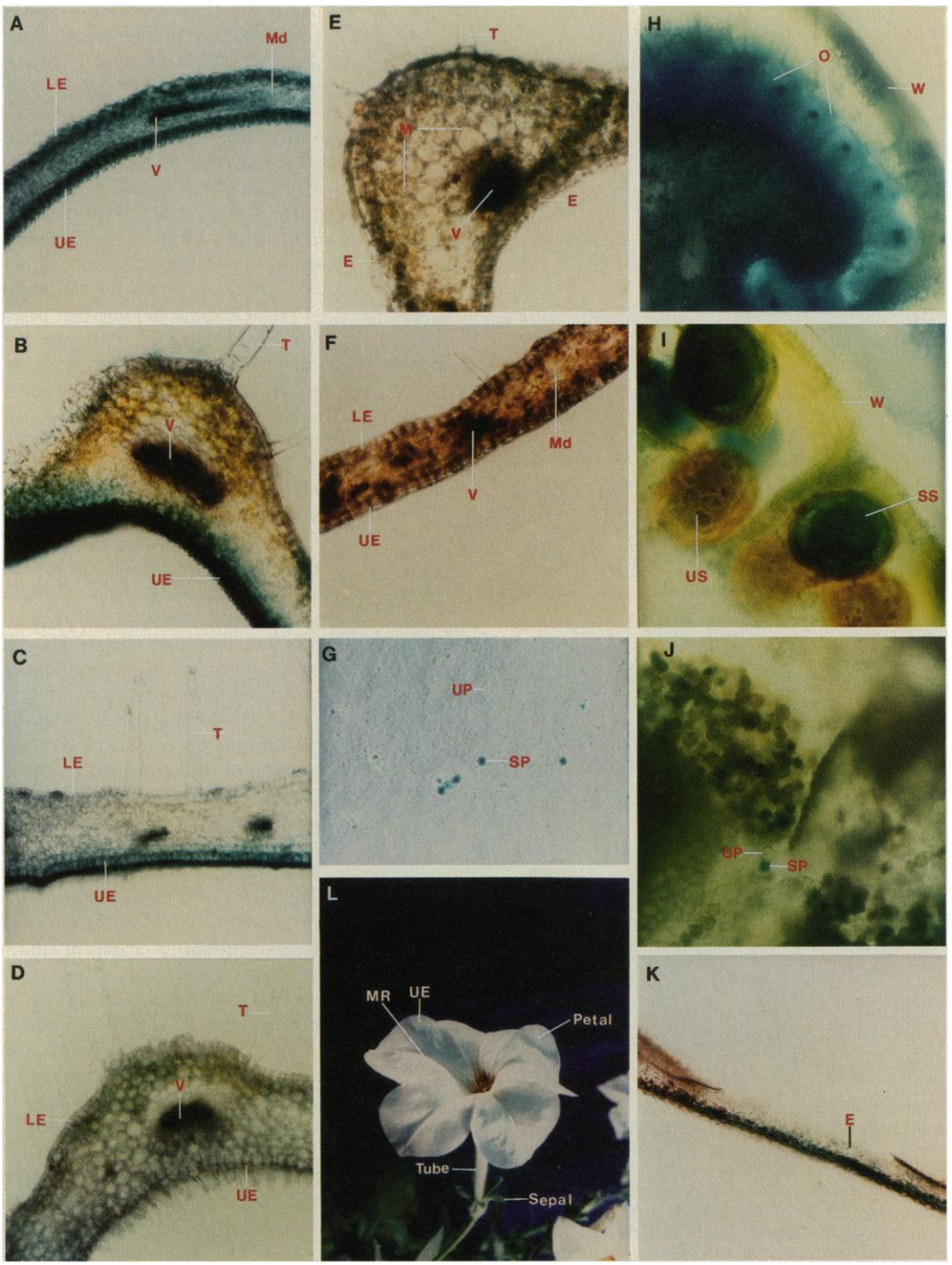

Fig. 3. Histochemical localization of GUS activity in transgenic petunia and tobacco floral tissue. Fresh sections were hand cut with a razor blade and incubated in a solution of 1 m*M* 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and 50 m*M* sodium phosphate buffer (*p*H 7.0) essentially as described (*46, 73*). Sections from sepals were incubated in 24-well microtiter dishes overnight at 37°C, then cleared of chlorophyll by incubation for 10 min in a solution of 5% formaldehyde, 5% acetic acid, and 20% ethanol, and then incubated for 2 min in 50% ethanol and 2 min in 100% ethanol, and washed twice in distilled water. The sections were mounted on microscope slides for photography. Sections from other tissues were incubated in the X-Gluc solution on microscope slides incubated overnight at 37°C in a humidified chamber. A Nikon Optiphot microscope with phase contrast optics was used for photomicrographs. Expression is from a construct containing the EPSP synthase promoter region from −1800 to −285 inserted in X-GUS-90 (*52*). Expression in transgenic petunia: (**A**) mature petal, (**B**) close-up of midrib of mature petal, (**C**) upper floral tube, (**D**) lower floral tube, and (**E**) sepal. Expression in transgenic tobacco: (**F**) mature petal, and (**G**) pollen. Developmental studies in petunia: (**H**) immature ovary from unopened flower, (**I**) seeds in maturing fruit, (**J**) pollen and anther, (**K**) petal from flower just after opening (obliquely cut section to show light blue staining in epidermis), and (**L**) petunia flower with anatomical parts indicated. Abbreviations: UE, upper epidermis; LE, lower epidermis; Md, mesoderm; V, vascular tissue; T, trichome; E, epidermis; M, mesophyll; UP, unstained pollen; SP, stained pollen; US, unstained seed; SS, stained seed; W, ovary wall; O, ovule; MR, midrib; and A, anther.

sequences to increase expression (*50*). Therefore, our ability to detect cell-specific expression from these constructs may be due in part to the use of this promoter element. Regulation of the EPSP synthase gene promoter differs quantitatively and perhaps qualitatively in different transgenic hosts. This is not unexpected in view of the difference in expression patterns of the endogenous genes in petunia and tomato (*49*). However, this finding emphasizes that for certain genes it is essential to use the homologous transgenic host in order to reproduce fully regulated expression. Previous work with an *Antirrhinum majus* chalcone synthase gene in tobacco (*54*), in which regulation was similar to that of the host plant and not the source, and an *N. plumbaginifolia* gene in petunia and in *N. plumbaginifolia* (*55*), in which the degree of light responsiveness differed in transgenic plants from the two species, supports this conclusion.

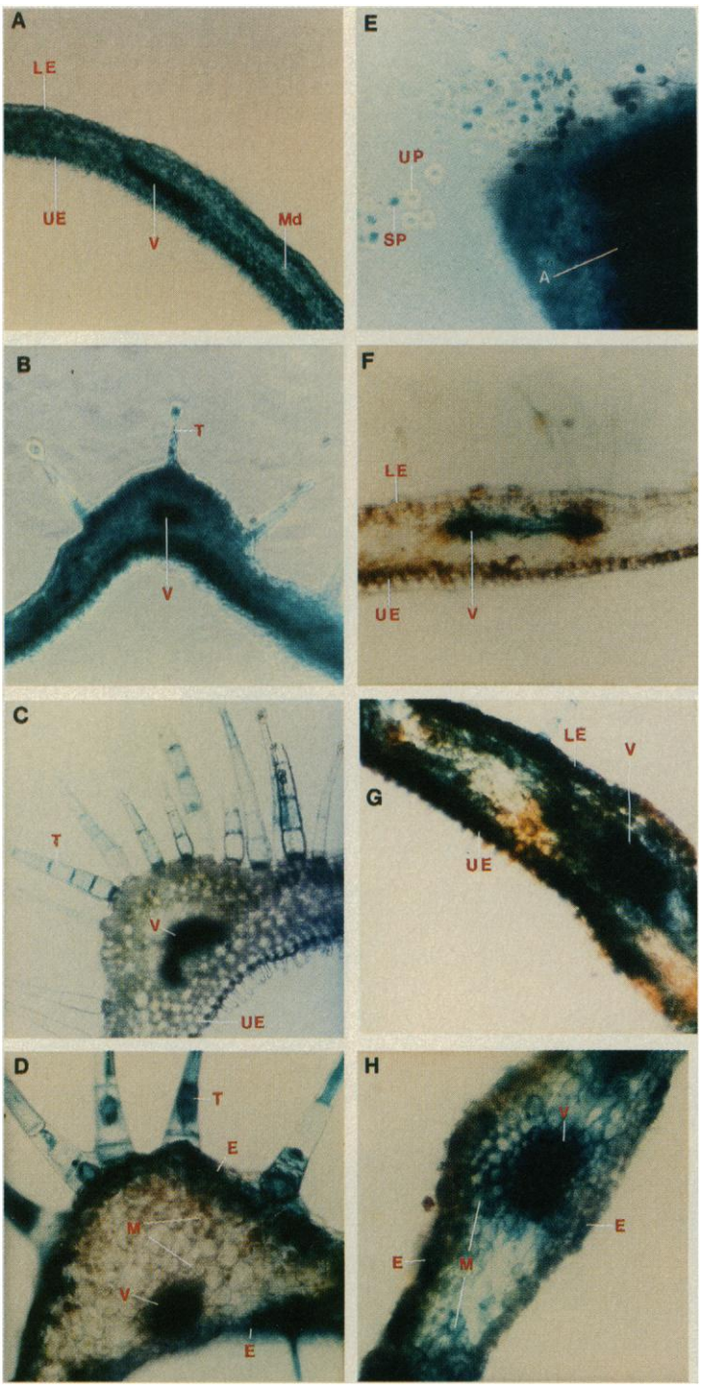

Fig. 4. Histochemical localization of expression from the nominally constitutive CaMV 35*S* promoter. Histochemical techniques were as described in Fig. 3. Expression is from the entire 35*S* promoter (−941 to +8) fused to the GUS coding sequence with an *rbc*S-3C 3′ end (*50*). Expression in transgenic petunia: (**A**) mature petal, (**B**) mature petal and midrib, (**C**) floral tube, (**D**) sepal, (**E**) anther and pollen. Expression in transgenic tobacco: (**F**) mature petal, (**G**) mature petal, and (**H**) sepal. See text for the number of plants assayed and the variation observed. Abbreviations as in Fig. 3.

Cell-Specific Expression of a Nominally Constitutive Promoter

As controls for these studies, we examined the cell specificity of expression of the nominally constitutive CaMV 35*S* enhancer (*56, 57*). The intermediate vector that contained the deletion constructs of the EPSP synthase gene promoter fused to the CAT coding sequence also contained a construct containing the entire 35*S* upstream region (−941 to +8) fused to a GUS coding sequence (*50*). In transgenic petunia, GUS activity was high in petals, but varied in leaves and stems from nearly background levels to levels almost as high as in petals. In transgenic tobacco, GUS activity in leaves and stems was high, and activity in petals was generally low (*51*). Similar results were obtained from constructs that did not contain the EPSP synthase gene construct in the same plasmid, ruling out possible cis effects of the EPSP synthase promoter on the 35*S* promoter as an explanation for these results (*51*).

Histochemical staining revealed an interesting pattern of expression of the 35*S*-GUS construct in floral tissue. In ten independent transgenic petunia plants, staining in petals was observed in all tissues (Fig. 4A), including the midrib (Fig. 4B) (compare to staining of the EPSP synthase construct in Fig. 3B). Variation in the staining pattern occurred in the tube, where staining was most frequently observed in the trichomes and in vascular tissue (Fig. 4C). This was in marked contrast to the EPSP synthase construct, where upper epidermis and adjacent cell layers stained (Fig. 3C). In sepals, one expression pattern we observed was staining only in the epidermis and trichomes (Fig. 4D). In all of these plants, pollen stained with an apparent 1:1 segregation ratio (Fig. 4E). We did not observe developmental regulation similar to that of the EPSP synthase construct. Petals just after opening were generally as intensely stained as mature petals (*51*). We observed a significant amount of variation among independent transgenic plants, particularly in the staining pattern of the tube and sepal. The most common pattern was vascular staining in both the tube and sepal, as well as staining of epidermis and trichome. In ten independent plants we never observed significant amounts of mesophyll staining in the sepal. In one plant, staining was confined to the vascular tissue in the petal, tube, and sepal (*51*).

In transgenic tobacco (15 independent plants) containing the 35*S*-GUS construct, staining of petals of mature flowers was principally observed in the vascular tissue (Fig. 4F), and occasionally, staining also appeared in epidermal and mesoderm tissue (Fig. 4G). The floral tube also showed vascular staining (*51*). Sepals showed vascular and mesophyll staining (Fig. 4H) (compare to petunia sepal in Fig. 4D). Variations in this pattern included one plant in which only trichomes stained and other plants in which trichomes (but not epidermis) and vascular tissue stained with equal intensity (*51*).

The use of histochemical localization to analyze gene expression is not without potential problems. In particular, accurate interpretation is dependent on such variables as the threshold of detection of the blue stain, cell size, and cell metabolic activity. However, the localization of GUS activity in the plants we assayed correlated well with CAT RNA and enzyme activity from plants that contained

equivalent chimeric promoter–CAT constructs. In addition, since the mRNA transcribed from the 35*S*-GUS construct should be identical to that from the chimeric EPSP synthase 35*S*-GUS construct, the striking differences in cell-specific expression observed (compare Figs. 3B and 4B, 3E and 4D, 3F and 4F) can be attributed to transcriptional regulation.

The sharp contrast in the predominant staining patterns of the 35*S*-GUS construct in the two transgenic hosts, best illustrated in the petals (compare Figs. 4A and 4F) and sepals (compare Figs. 4D and 4H) indicates that when expression at the level of individual cells is assayed, a promoter that appears to be constitutive can show striking specificity of expression. From this we conclude that the 35*S* promoter does not confer constitutive expression in all cell types. Similar observations were made for expression of a 35*S*-GUS construct in the stem of transgenic tobacco (*46*). Although variation among individual transgenic plants within species has been frequently observed and attributed to differences in the position of insertion in the chromosome (*56, 58*), variation in tissue-specific expression patterns between transgenic hosts is more difficult to explain.

One model that can explain the present findings draws on work with the simian virus 40 (SV40) large T promoter in animal cells. Constitutive expression of this promoter in various cell lines is due to the presence of multiple sequence elements, each with a different specificity for different cell types (*59*). If the 35*S* promoter is similarly constituted, it is possible that elements conferring expression in specific cell types within a transgenic plant are affected differently by chromosomal position. In petunia, for example, since the three staining patterns we observed in the sepal are (i) epidermis and trichome; (ii) epidermis, trichome, and vascular tissue; and (iii) only vascular tissue, sequences that control expression in the epidermis and trichome may be affected differently from sequences that control expression in vascular tissue. Similar variations in tissue-specific expression that are dependent on the position of insertion of the transgene were found for the α-fetoprotein gene in transgenic mice (*60*). Variation in expression patterns between tobacco and petunia can likewise be explained by relative differences in expression governed by these sequences. The factors that recognize individual sequence elements in the 35*S* promoter and confer cell-specific expression may differ in abundance or affinity in the two different plants. Experiments to test this hypothesis are under way.

Conclusion

We used transgenic plants to define sequences responsible for cell-specific and developmentally regulated expression of the EPSP synthase gene. The ability to detect cell-specific expression in a transgenic plant permitted us to redefine the regulation of the CaMV 35*S* promoter previously thought to be constitutive. This level of resolution may allow for the identification of sequences which, as individual elements (or in some combination), are responsible for expression in specific cell types. Identification of such elements and the trans-acting factors that interact with them is essential for understanding the gene regulation that governs development in higher plants.

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