The Plant Cell, Vol. 2, 849–856, September 1990 © 1990 American Society of Plant Physiologists

Sequence Requirements of the 5-Enolpyruvylshikimate-3-phosphate Synthase 5′-Upstream Region for Tissue-Specific Expression in Flowers and Seedlings

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We have analyzed expression from deletion derivatives of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) 5′-upstream region in transgenic petunia flowers and seedlings. In seedlings, expression was strongest in root cortex cells and in trichomes. High-level expression in petals and in seedling roots was conferred by large (>500 base-pair) stretches of sequence, but was lost when smaller fragments were analyzed individually. This apparent requirement for extensive sequence suggests that combinations of *cis*-elements that are widely separated control tissue-specific expression from the EPSPS promoter. We have also used the high-level, petal-specific expression of the EPSPS promoter to change petal color in two mutant petunia lines.

INTRODUCTION

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) catalyzes an essential step in the shikimate pathway leading to the biosynthesis of aromatic amino acids and chorismate-derived secondary metabolites. These secondary metabolites include lignins, flavanols, and anthocyanins. EPSPS is also the target for the broad-spectrum herbicide glyphosate. An initial study of the endogenous EPSPS gene in petunia revealed a complex expression pattern (Gasser et al., 1988). In mature petunia plants, expression is very high in petals and barely detectable in leaves (Gasser et al., 1988). In seedlings, expression is strongest in roots and stems but lower than in petals (Gasser et al., 1988). We have undertaken an analysis of the sequences required for regulated expression of the EPSPS gene. The complexity of the expression pattern—expression in different organs at different stages of development—suggested that this gene might provide insight into the regulation of genes whose expression is required in different tissues and at more than one stage of development.

We showed previously that high-level expression in petunia petals can be conferred by upstream sequences of the petunia EPSPS gene (Benfey and Chua, 1989). A preliminary deletion analysis indicated that sequences between −1800 and −800 are sufficient to confer this tissue-specific expression. Expression from the transgene is also regulated during floral development with activity increasing dramatically as the flower opens. Histochemical analysis revealed that expression is in nearly all cell types of the limb of the flower and restricted to cells adjacent to and in the upper epidermis in the upper portion of the tube (Benfey and Chua, 1989). We have now analyzed a more detailed set of deletion derivatives that reveal functional redundancy for expression in petunia petals. We defined the tissue-specific expression conferred by these deletion constructs in seedlings. In addition, we used the maize A1 gene as a phenotypic reporter gene to monitor expression conferred by the upstream EPSPS sequences. The requirement for extensive sequence for high-level petal and seedling root expression suggests that combinations of *cis*-elements with binding sites for the same or different *trans*-factors may control tissue-specific expression from the EPSPS gene.

RESULTS

Sequence and Constructs

Deletion derivatives of the EPSPS 5′-upstream region were generated by cutting at convenient restriction sites (see Methods). Figure 1 shows the endpoints and orientation of the fragments used in this analysis. We analyzed expression from all fragments to detect functional redundancy. In construct 1, the fragment from −1752 to −30 was placed upstream of the EPSPS TATA region (−63 to +3), which was fused to the β-glucuronidase (GUS) coding

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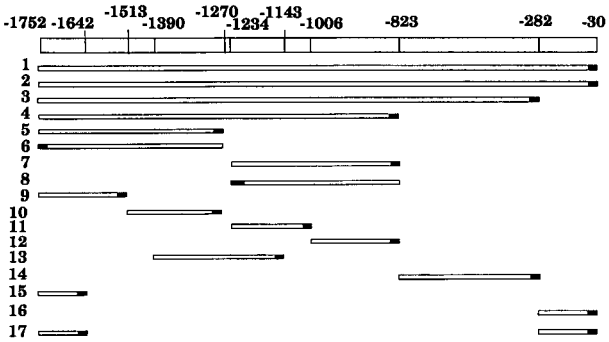

Figure 1. Fragments from the EPSPS 5′-Upstream Region Used in Expression Constructs.

The sequence coordinates are given above the schematic representation of the EPSPS 5′-upstream region. The orientation of each fragment is indicated by the shaded box at one end of the fragment. The construct number is given to the left of the fragment included in that construct.

sequence with a 3′ polyadenylation sequence from the pea *rbc*S 3C gene. In constructs 2 to 17, the fragments were inserted in the expression vector X-GUS-46 described by Benfey et al. (1990b). This expression vector contains a polylinker upstream of the cauliflower mosaic virus (CaMV) 35S −46 to +8 TATA region fused to the GUS coding sequence with a 3′ polyadenylation sequence from the pea *rbc*S 3C gene. The constructs were transferred into petunia and analyzed for expression of the GUS reporter gene.

Expression in Flowers

Expression was initially analyzed by the fluorimetric assay of GUS enzymatic activity in flowers and leaves of the primary transformants. In leaf tissue, we detected no activity significantly above background from any of the constructs. The vector alone (X-GUS-46) conferred no detectable expression in petal or leaf tissue. Table 1 presents the results from tissue extracts of the limb of mature flowers. High-level GUS activity was observed in plants containing constructs 1 to 4, which have 3′ deletion endpoints at −30, −282, and −823. Further deletion to −1270 (construct 5) resulted in a significant reduction in the highest level of GUS activity recorded. Moreover, functional redundancy for expression was indicated by approximately equal levels of expression for construct 7 (−1234 to −823) as for construct 5 (−1752 to −1270). The fragment from −1234 to −823 was clearly active in both orientations (constructs 7 and 8), whereas only one plant

Table 1. β-Glucuronidase Enzymatic Activity in Independent Transgenic Plants Containing Different EPSPS Constructs

Construct	TATA	>20,000	20,000 to 10,000	10,000 to 2000	2000 to 0	Total
1 (−1752 to −30)	EPSPS	5	3	5	2	15
2 (−1752 to −30)	35S	7	2		2	11
3 (−1752 to −282)	35S	3	2	2	5	12
4 (−1752 to −823)	35S	2	3	2	4	11
5 (−1752 to −1270)	35S			6	4	10
6 (−1752 to −1270)R	35S			1	4	5
7 (−1234 to −823)	35S			5	4	9
8 (−1234 to −823)R	35S			3	8	11
9 (−1752 to −1513)	35S				9	9
10 (−1513 to −1270)	35S				7	7
11 (−1234 to −1006)	35S				7	7
12 (−1006 to −823)	35S			1	11	12
13 (−1390 to −1143)	35S				10	10
14 (−823 to −282)	35S				9	9
15 (−1752 to −1642)	35S				9	9
16 (−282 to −30)	35S		1	2	10	13
17 (−1752 to −1642 and −282 to −30)	35S				13	13
X-GUS-46	35S				10	10

The construct number (from Figure 1) and sequence coordinates are given in the first column. The TATA region used is given in the second column. The EPSPS TATA region used was from −63 to +3, the 35S TATA region was from −46 to +8. In columns 3 to 6 the number of independent transgenic plants for which the fluorimetric assay of GUS activity was in the range indicated above the column is given. GUS activity is for tissue from the limb of mature petunia flowers and is given in picomoles of 4-methylumbelliferone per milligram of protein per minute. In column 7 the total number of transgenic plants analyzed for each construct is given. R, reverse orientation.

with the fragment −1752 to −1270 was active in the reverse orientation (construct 6). Subdivision of the two 0.5-kb fragments gave four fragments (−1752 to −1513, −1513 to −1270, −1234 to −1006, and −1006 to −823) with greatly diminished activity (constructs 9 to 12). Only a single plant with construct 12 (−1006 to −823) showed any significant expression in petals. We made construct 13 (−1390 to −1143) so that it spanned the junction between the 500-bp fragments. This fragment was also unable to confer activity in petunia petals.

Although the 3′ deletion analysis suggested that the sequences that control petal-specific expression are upstream of −823, we tested the downstream sequences as well to detect further functional redundancy. The fragment from −823 to −282 (construct 14) gave no expression above background levels in petal or leaf tissue. We also tested fragments containing the sequences from −1752 to −1642, −282 to −30, and the combination of these two fragments. The fragment from −1752 to −1642 (construct 15) showed no ability to confer expression in petunia petals. Three plants containing the fragment from −282 to −30 (construct 16) showed expression in petals. Two plants were slightly above background levels, and a third plant was significantly above background levels but its expression in seedling root tissue was aberrant (see below), raising the possibility that expression of the transgene in this plant is strongly influenced by nearby chromosomal sequences. The combination of the fragments −1752 to −1642 and −282 to −30 (construct 17) was also inactive by this assay in petals.

We conclude that high-level, petal-specific expression requires more than 0.5 kb of EPSPS upstream sequence. Lower-level, petal-specific expression can be conferred by two different regions, at least one of which acts in an orientation-independent manner, indicating the presence of functionally redundant sequences. Expression in petals is reduced to undetectable levels when these two fragments are cut in two, suggesting either that critical *cis*-elements are located precisely at the breakpoints or that a combination of sequences on either side of the breakpoint is necessary to confer regulated expression.

Histochemical Analysis of Expression in Petals

We performed histochemical analysis of expression on a subset of plants containing the EPSPS/GUS chimeric constructs to answer the following questions: (1) Does the choice of minimal promoter significantly affect expression conferred by the EPSPS upstream sequences in petals? (2) Is the decrease in GUS activity observed with constructs 5 and 7 due to a change in the cell specificity of expression or to decreased expression in all cell types that show expression with the longer fragments? (3) Can we detect any differences in cell-specific expression with constructs 5 and 7 that are functionally redundant for expression in petals as determined by the fluorimetric assay?

In our previous analysis, we used the cauliflower mosaic virus 35S −90 to +8 promoter for the histochemical analysis of the EPSPS 5′-upstream sequence (Benfey and Chua, 1989). We have now analyzed the expression pattern conferred by the EPSPS upstream region with the 35S −46 to +8 promoter and the EPSPS −63 to +3 promoter. The cell-specific expression pattern in petals that we observed from constructs with 3′ endpoints at −30, −282, and −823 fused to the 35S −46 to +8 fragment (constructs 2 to 4) was identical to that of the −1752 to −282 fragment fused to the 35S −90 to +8 promoter. Figure 2A shows expression from construct 3 (−1752 to −282 with the −46 promoter) in mature petunia petals. GUS staining was detected in both epidermal layers as well as in the mesophyll and vascular cells. Expression in the floral tube was also identical to that found with the −90 to +8 promoter, with an apparent gradient of expression from the top to the bottom of the tube. At the top of the tube, expression was observed in the upper epidermis and adjacent mesophyll cells (Figure 2B). Lower in the tube, expression was only detected in the upper epidermis, and at the base of the tube, no expression was observed. This expression pattern was also identical to that found with a fragment from −1752 to −30 fused to the EPSPS TATA region from −63 to +3 (construct 1). The quantitative analysis shown in Table 1 also indicates that there was little difference when the 35S TATA sequence was substituted for the EPSPS TATA sequence. In addition, the activation of expression during flower opening appeared to be identical for all three TATA regions. Figure 2C shows that in a flower just after opening expression was only detectable in the upper epidermis. This flower was from the same plant as the flower in Figure 2A. Expression with all three TATA regions was undetectable in sepals.

The cell-specific expression conferred by construct 5 (−1752 to −1270) and construct 7 (−1234 to −823) appeared to be lower but in the same cell types as from the longer fragments. Figure 2D shows expression in the petal from construct 7. In general, expression was lower in the limb of these plants and was rarely detected in any tissue in the tube. We did not observe any consistent difference in expression between constructs 5 and 7, nor were we able to detect any expression in a specific cell type in petals from plants containing constructs 9 to 12.

We conclude that the principal determinants of the cell-specific expression pattern in flowers reside in the upstream sequences and not in the TATA region. Even addition of the 35S region between −90 and −46 that can have significant effects on cell specificity when combined with other *cis*-elements (Benfey et al., 1990a, 1990b) appears to have no effect on the cell specificity of expression in floral tissue conferred by the EPSPS sequences. The functional redundancy that we observed with the GUS fluorimetric assay appears to exist also at the level of cell specificity.

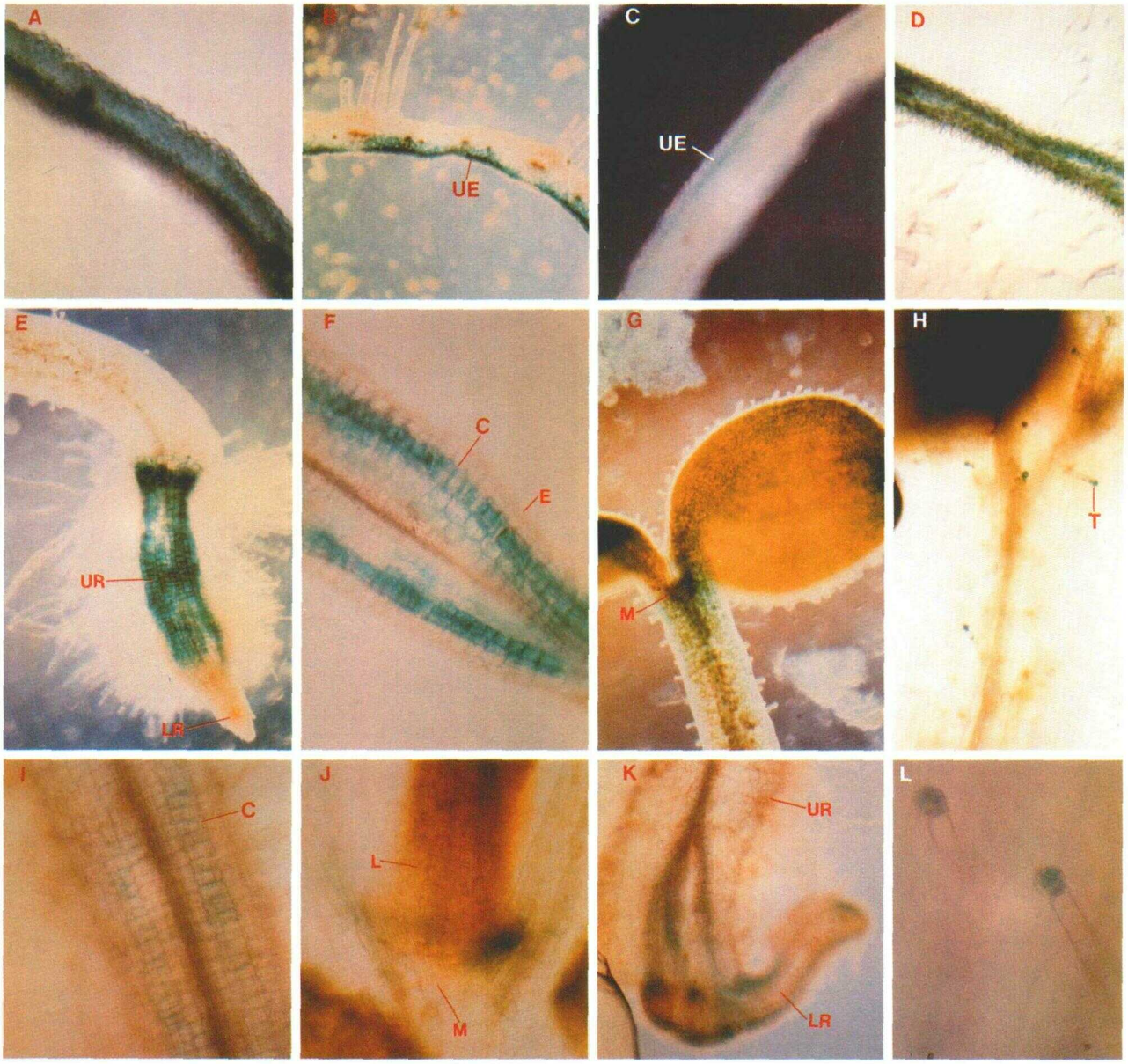

Figure 2. Histochemical Analysis of Expression Conferred by EPSPS Constructs in Petunia Petals and Seedlings.

(A) Expression from construct 3 (−1752 to −282) in the limb of a mature flower.
(B) Expression from construct 3 in the tube of a mature flower.
(C) Expression from construct 3 in the limb of a flower from the same plant as **(A)** but just after the flower opened.
(D) Expression from construct 7 (−1234 to −823) in the limb of a mature flower.
(E) Expression from construct 2 (−1752 to −30) in a 10-day-old seedling.
(F) Higher magnification of the root of a seedling containing construct 2.
(G) Aerial portions of a 10-day-old seedling containing construct 4 (−1752 to −823).
(H) Expression from construct 2 (−1752 to −30) in a 10-day-old seedling showing expression in stem trichomes.
(I) Expression from construct 5 (−1752 to −1270) in the root of a 10-day-old seedling.
(J) The stem apex region of a 10-day-old seedling containing construct 13 (−1390 to −1143).
(K) Expression from construct 13 in the root of a 10-day-old seedling.
(L) High magnification of stem trichomes from a 10-day-old seedling containing construct 4 (−1752 to −823).
C, cortex; E, epidermis; L, leaf; LR, lower portion of root; M, meristematic region; T, trichome; UE, upper epidermis of limb; UR, upper portion of root.

Expression in Seedling

Gasser et al. (1988) reported previously that the endogenous petunia EPSPS gene shows elevated expression in roots and stems of seedlings. We have used histochemical analysis to identify the cell types in seedlings responsible for this elevated expression. Seeds from plants containing the EPSPS/GUS chimeric constructs were allowed to germinate in media containing antibiotics for selection. Seedlings were removed at 7 days, 10 days, and 17 days and stained as whole mounts as described previously (Benfey et al., 1989).

Expression from constructs 1 to 4 with endpoints at −30, −282, and −823 was strongest in the root (Figure 2E). Expression appeared to be principally in the cortex tissue (Figure 2F). In most plants, expression was not detected in the lower portion of the root (Figure 2E). With constructs 1 to 4, some expression in the meristematic region of the stem (Figure 2G) and in the mesophyll cells of cotyledons and true leaves was observed. In addition, expression was frequently observed with these constructs in trichomes of stem (Figures 2H and 2L) and leaf. In plants containing the two 500-bp fragments (constructs 5 and 7), the only expression detected was in root cortex tissue, but expression was quite weak (Figure 2I).

Among the smaller fragments, constructs 9, 10, and 11 gave no expression in any tissue at any stage. Construct 12 (−1006 to −823), however, conferred weak expression in two plants in root cortex. Construct 13 (−1390 to −1143), which spans the deletion between −1234 and −1270 (see Figure 1), was able to confer weak expression at the base of true leaves (Figure 2J) and in root cortex (Figure 2K). The root expression was usually in the lower portion of the root (Figure 2K). No expression in trichomes was observed with any of these constructs. One plant with construct 16 (−282 to −30) showed weak root cortex expression. The plant with construct 16 that had moderately high expression in petals (15,000 pmol of 4-methylumbelliferone/mg of protein/min) showed expression principally in the root cap. This expression pattern was not observed with any of the other transformants, suggesting that sequences at the site of insertion may have influenced the expression pattern. One plant with construct 17 (−1752 to −1642 and −282 to −30) showed weak expression in root cortex and very weak expression in stem trichomes. Table 2 shows the numbers of independent transgenic plants with these staining patterns.

We conclude from the histochemical data that expression in seedling conferred by the upstream regions of the EPSPS gene is principally in root cortex tissue, with some expression also in trichomes and in the stem meristematic region. As with petal-specific expression, strong expression in seedling root appears to require more than 0.5 kb of sequence. A smaller fragment (−1390 to −1143) is able to confer expression in root tissue; however, the pattern of expression (in lower root versus upper root) suggests that there may be more than one region required for high activity in root.

Table 2. Histochemical Analysis of Petunia Seedlings Containing EPSPS 5′-Upstream Constructs

Construct	TATA	Seedling Root Cortex	Seedling Trichome	Total Analyzed
1 (−1752 to −30)	EPSPS	5/5	4/5	5
2 (−1752 to −30)	35S	7/9	6/9	9
3 (−1752 to −282)	35S	3/6	0/6	6
4 (−1752 to −823)	35S	3/4	2/4	4
5 (−1752 to −1270)	35S	1/6	0/6	6
7 (−1234 to −823)	35S	2/3	0/3	3
8 (−1234 to −823)R	35S	6/6	0/6	6
9 (−1752 to −1513)	35S	5/5	0/5	5
10 (−1513 to −1270)	35S	0/5	0/5	5
11 (−1234 to −1006)	35S	0/3	0/3	3
12 (−1006 to −823)	35S	2/7	0/7	7
13 (−1390 to −1143)	35S	6/7	0/7	7
16 (−282 to −30)	35S	1/9	0/9	9
17 (−1752 to −1642 and −282 to −30)	35S	1/6	1/6	6

The construct number (from Figure 1) and sequence coordinates are given in the first column. The TATA region used is given in the second column (see Table 1). The number of independent transgenic plants with GUS histochemical staining in seedling root cortex among the total number of plants analyzed is given in column 3. In column 4, the number of independent transgenic plants with GUS histochemical staining in seedling trichomes among the total number of plants analyzed is given. For details of staining patterns see text and Figure 2. The total number of independent transgenic plants analyzed for each construct is given in column 5. R, reverse orientation.

Changing Petal Color with the EPSPS Promoter

Meyer et al. (1987) showed previously that expression of the maize A1 gene encoding dihydroquercetin 4-reductase in a petunia mutant that accumulates dihydrokempferol results in formation of pelargonidin pigments not normally found in petunia. The coding sequence of the A1 gene was driven by the 35S promoter in the study by Meyer et al. We wished to determine whether the maize A1 gene could be used as a phenotypic reporter gene for expression conferred by the EPSPS upstream sequences and whether the developmental kinetics of EPSPS gene activation would result in a different pattern of pigment appearance. We fused the EPSPS upstream sequences from −1752 to −130 to the 35S −46 to +8 promoter upstream of the maize A1 coding sequence and introduced the chimeric gene into two different mutant petunia lines. The RL01 line used in the study by Meyer et al. is a regulatory mutant that produces a small amount of delphinidins and

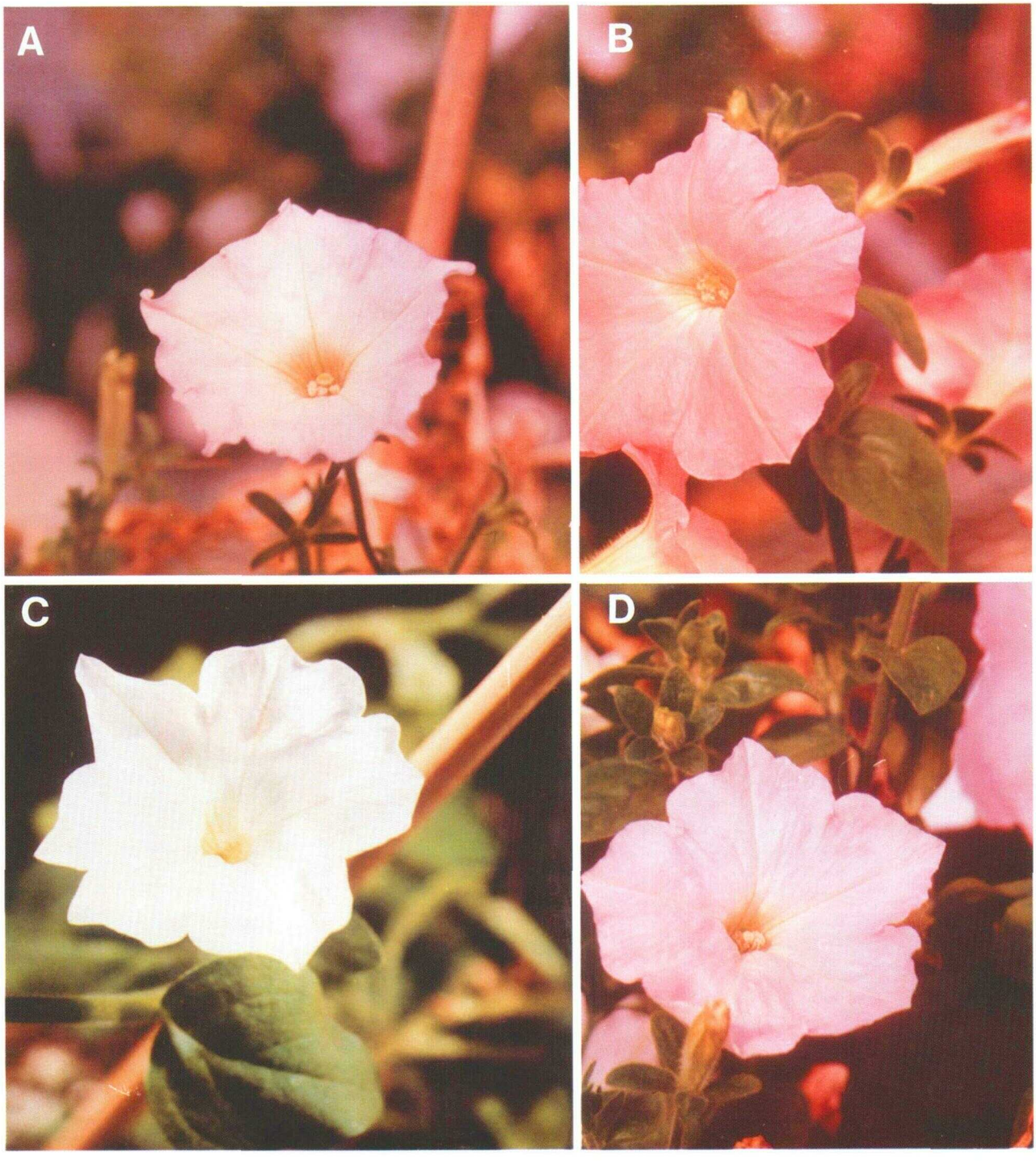

Figure 3. Changing Petal Color with the EPSPS 5′-Upstream Region Fused to the Maize A1 Coding Sequence.

(A) A flower from the mutant petunia line RL01.
(B) Expression from a chimeric EPSPS/A1 gene in the RL01 line.
(C) A flower from the mutant petunia line W80.
(D) Expression from a chimeric EPSPS/A1 gene in the W80 line.

cyanidins (Figure 3A). The W80 mutant is completely lacking visible anthocyanin pigments (Figure 3C). The chimeric EPSPS/A1 gene was able to cause pigment accumulation in both mutant lines. In the RL01 line, a uniform pink-orange color was produced (Figure 3B). In the W80 line a lighter pink color was produced (Figure 3D). In both cases pigment was detectable in the developing buds before the flower opened. This may be due to the clearly detectable levels of EPSPS expression in the developing bud that are sufficient to produce visible pigments.

DISCUSSION

We have provided evidence that the minimal EPSPS 5′ sequence required for high-level expression in petunia petals is greater than 500 bp. Tissue-specific expression at lower levels is conferred by fragments of approximately 500 bp. The observation that two different 5′ fragments are able to confer nearly identical expression patterns indicates that there is functional redundancy for expression. We note that if we had performed a normal deletion analysis rather than testing all fragments, we would have missed the fact that both fragments are able to confer tissue-specific expression. Because there was only a quantitative decrease in the level of expression with the 3′ deletion from −823 to −1270, one possible interpretation was that the *cis*-elements within this fragment do not play a role in conferring tissue-specific expression. However, when we tested the isolated fragment (−1234 to −823), we observed that it was able to confer tissue-specific expression. In addition, this fragment is able to work in an orientation-independent manner, which is one of the characteristics of enhancer sequences.

We have also shown that when the two 500-bp fragments are cut in two, none of the fragments alone is able to confer regulated expression. One possible explanation for these results is that sequences at the breakpoints used to separate the fragments are critical for high-level expression. We tested this hypothesis for the loss of expression when the larger fragment was cut into two 500-bp fragments by using a 247-bp fragment that spanned the breakpoint at −1234. We observed no expression at all in petals with this fragment. An alternative explanation is that high-level tissue-specific expression is conferred by a combination of *cis*-elements. Thus, the lack of expression from the smaller fragments could be due to the need for numerous *cis*-elements that bind the same or different *trans*-factors. In support of this hypothesis, we have characterized a factor found in nuclear extracts from petunia petals that binds specifically to numerous sites on the EPSPS promoter (H. Takatsuji, P. Benfey, and N.-H. Chua, unpublished observations).

Our histochemical analysis of expression conferred by the EPSPS 5′ sequence in petunia seedlings revealed that expression was strongest in the cortical cells of the root. The sequence requirements for root expression would appear to be similar to those for petal expression. However, the fragment from −1390 to −1143 is able to confer expression in root, but no expression is detected in petal. This fragment alone is able to confer only low-level expression in roots, and the expression is principally in the lower portion of the root. It appears, therefore, that combinations of *cis*-elements may also be important for the expression in seedling roots.

We have given evidence for the ability of the EPSPS upstream region to confer expression in specific tissues of seedlings and flowers. The physiological reason for an increased level of expression in these tissues is still unclear. However, understanding how expression from a complex promoter is regulated in different tissues and at different stages of development should provide insight into the regulation of a large class of genes that are expressed at more than one time in the development of the organism.

METHODS

Constructs

The DNA sequence of the EPSPS upstream region from pMON9561 (Gasser et al., 1988) was determined from the EcoRI site at −1752 to the SalI site at −282 by the dideoxy chain-termination method (Sanger et al., 1977) after subcloning into derivatives of pEMBL plasmids. The sequence has been deposited in the GenBank with accession number M37029. Fragments of the EPSPS upstream region were cleaved at convenient restriction sites and subcloned into derivatives of pEMBL vectors. Orientation and fidelity of cloning were verified by digesting with restriction enzymes that cut in internal sites. The sites used were as follows: construct 1, EcoRI to DraI (at −30); construct 2, EcoRI to DraI (at −30); construct 3, EcoRI to SalI; construct 4, EcoRI to AccI; construct 5, EcoRI to SspI (at −1270); construct 7, SspI (at −1234) to AccI; construct 9, XmnI to StuI; construct 10, StuI to SspI (at −1270); construct 11, SspI (at −1234) to XmnI; construct 12, XmnI to AccI; construct 13, HincII (at −1390) to HincII (at −1143); construct 14, AccI to SalI; construct 15, EcoRI to XbaI; construct 16, SalI to DraI (at −30); construct 17, EcoRI to XbaI and SalI to DraI (at −30). Except where noted, the sites are unique. For constructs 2 to 17, the subcloned fragments were then inserted between the HindIII and XhoI sites of the polylinker of X-GUS-46. For construct 1, a fragment from −63 to +3 of the EPSPS promoter was synthesized as complementary oligonucleotides, subcloned into a pEMBL derivative, and sequenced for accuracy. The fragment was then ligated into the X-GUS-0 vector. This vector is nearly identical to X-GUS-46 except that the polylinker is immediately upstream of the GUS coding sequence. Subsequently, the EPSPS fragment from −1752 to −30 was inserted upstream of the −63 to +3 region. For the EPSPS/A1 construct, the maize A1 cDNA described by Meyer et al. (1987) was inserted into the binary vector pMON505 (Horsch and Klee, 1986) with a 3′ end from the pea *rbc*S-E9 gene. Upstream of the A1, cDNA was inserted the EPSPS upstream region from −1752 (EcoR1 site) to −130 (PvuII site) fused to the 35S −46 to +8 TATA region.

Fluorimetric Analysis

Fluorimetric analysis of GUS activity was performed as described by Benfey et al. (1989), which is a modification of the method of Jefferson et al. (1987). Limb tissue from three flowers at different developmental stages (just before opening, opened but before pollen release, opened after pollen release) and leaf tissue were analyzed. Five micrograms of protein were incubated with the substrate 4-methylumbelliferyl glucuronide for 1 hr as described by Benfey et al. (1989). Fluorescence was measured with a Perkin-Elmer LS5 fluorimeter. Fluorescence of a solution of 100 nM 4-methylumbelliferone in 0.2 M sodium carbonate was used for calibration. In Table 1, we present the results for limb tissue from flowers opened after pollen release. In general, the flowers at the earlier developmental stages gave lower enzymatic activity, in agreement with the increase in expression of the EPSPS gene as the flower opens (Benfey and Chua, 1989).

Histochemical Analysis

Histochemical analysis was performed on floral material as described by Benfey and Chua (1989). Two flowers from two to five independent transgenic plants were hand sectioned and analyzed for expression in limb, tube, and sepal. Figure 2 shows representative sections. Histochemical analysis on seedlings was performed as described by Benfey et al. (1989). The number of plants analyzed is given in Table 2.

ACKNOWLEDGMENTS

We thank Dr. Anton Gerats for the gift of the W80 petunia line, Drs. Heinz Saedler and Peter Meyer for the gift of the RL01 petunia line and the maize A1 gene, Charles Koo for expert technical assistance, and members of the Chua laboratory for many helpful discussions. P.N.B. was a fellow of the Helen Hay Whitney Foundation. This work was supported by a grant from the Monsanto Company.

Received June 1, 1990; revised July 11, 1990.

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