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Developmental and UV Light Regulation of the Snapdragon Chalcone Synthase Promoter

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Expression directed by the 1.1-kb snapdragon chalcone synthase (CHS) promoter linked to the β-glucuronidase reporter gene has been studied in transgenic tobacco. The pattern of expression of the chimeric gene was compared with the expression of the endogenous CHS genes in tobacco and snapdragon. We demonstrate that expression of the CHS promoter is controlled in both an organ-specific and tissue-specific manner. The highest level of expression was observed in immature seeds. Deletions were used to define regions of the promoter required for expression in roots, stems, leaves, seeds, and flower petals of transgenic plants. We have defined the minimal sequences required for expression in different organs and mapped regions of the promoter that influence expression in either a positive or negative manner. A promoter fragment truncated to −39 activates transcription in roots of 4-week-old seedlings, whereas a fragment extending to −197 bp directs expression in petals and seeds. A positive regulatory element located between −661 and −566 and comprising a 47-bp direct repeat is active in all tissues investigated except petals. UV light-regulated expression in leaves of transgenic tobacco seedlings is dependent on the presence of sequences also required for leaf-specific expression. Within the intact promoter, sequences that individually confer different patterns of expression interact to produce the highly regulated expression pattern of CHS.

INTRODUCTION

Chalcone synthase (CHS) catalyzes the first step of the flavonoid-specific branch of the phenylpropanoid pathway. Flavonoids serve a variety of different and apparently unrelated functions in plants. In addition to being involved in pigmentation of flowers and fruits, flavonoids have been implicated in contributing to protection from UV light irradiation and the defense of the plant in response to pathogens as well as wounding (for recent review, see Dangl et al., 1989). Moreover, flavonoids play a role in the symbiotic nodulation process between legumes and nitrogen-fixing bacteria (Peters et al., 1986). These ideas are supported by the changes in CHS activity observed during plant development (Schmelzer et al., 1988; Koes et al., 1989) as well as in response to UV light (Chappell and Hahlbrock, 1984) and wounding (Lawton and Lamb, 1987). Generally, the increases in CHS activity have been correlated with elevated steady-state levels of CHS mRNA. Nuclear runoff experiments using parsley cells exposed to UV light have indicated that the accumulation of CHS mRNA is controlled primarily at the transcriptional level (Chappell and Hahlbrock, 1984).

In some species such as petunia, French bean, and soybean, CHS is encoded by a small multigene family (Koes et al., 1987; Ryder et al., 1987; Wingender et al., 1989). In French bean, there is differential expression of individual gene family members in response to differing stimuli (Ryder et al., 1987), whereas in soybean expression of a single gene can be induced by both UV light and fungal elicitor (Wingender et al., 1989). In contrast, parsley, snapdragon, and Arabidopsis apparently have a single CHS gene (Sommer and Saedler, 1986; Feinbaum and Ausubel, 1988; Herrmann et al., 1988). Hence, it is likely that in the latter cases the CHS promoter responds to a variety of environmental and developmental cues triggering transcription.

Previously, two general approaches have been used to study the *cis* regulatory elements from the snapdragon CHS promoter. One approach used the insertion and imprecise excision of transposable elements in vivo, whereas the other utilized deletions generated in vitro that were then linked to marker genes and reintroduced into plant cells. Transposon insertions between −70 and −50 of the promoter lead to a decrease in CHS expression and pigment intensity in the flower (Sommer and Saedler, 1986). Partial revertants, missing a portion of a repeat sequence located between −55 and −43, also show a decrease in CHS mRNA (Sommer et al., 1988). In the

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alternate approach, a 1.1-kb CHS promoter fragment from snapdragon, linked to a neomycin phosphotransferase (NPTII) marker gene, was found to be sufficient for white light induction in dark-adapted tobacco teratoma tissue and UV light induction in the leaves of tobacco seedlings (Kaulen et al., 1986). Moreover, 5′ end deletions of this promoter fragment demonstrated the presence of at least two regulatory regions. One region spanning position −1050 to −357 is essential for maximal expression, whereas sequences located between −357 and −39 are required for white light induction. Expression directed by CHS promoter fragments fused to NPTII in the UV light-inducible parsley protoplast transient expression system revealed that the region from −197 to −39 functions as an orientation independent, UV light-responsive element (Lipphardt et al., 1988). In addition, this region is able to render a heterologous minimal promoter UV light responsive. Sequences between −357 and −197, although by themselves insufficient for UV light induction, specifically potentiate the level of UV light-induced expression when combined with the UV light-responsive element. Moreover, an enhancer-like sequence located between −661 and −564 was identified (Lipphardt et al., 1988). This region contains a 47-bp direct repeat sequence that bears similarities to viral enhancer sequences (Kaulen et al., 1986).

We were interested in studying transgenic tobacco containing snapdragon CHS promoter derivatives linked to a marker gene to further characterize the *cis* regulatory sequences controlling expression of the promoter. Using fusions to the β-glucuronidase (GUS) marker gene (Jefferson et al., 1987), we demonstrate that the promoter can direct tissue-specific as well as developmentally regulated expression. We compare these patterns of expression with that of the endogenous CHS genes of both snapdragon and tobacco. Using deletion derivatives, we define the regions of the promoter that are important in directing the expression in different plant tissues at differing developmental stages and characterize regions responsible for UV light inducibility in the leaves of transgenic tobacco seedlings.

RESULTS

Expression of CHS in Snapdragon and Tobacco

Patterns of accumulation of CHS mRNA in different organs of tobacco and snapdragon were tested to effectively interpret the behavior of the snapdragon CHS promoter constructs in transgenic tobacco. To do this, total RNA was quantitatively extracted from differing organs of both plants at comparative stages of development and subjected to RNA dot blot analysis using a hybridization probe derived from either a tobacco genomic CHS clone (pCS1, kindly provided by Dr. Gary Drews, California Institute of Technology, Pasadena) or a snapdragon CHS cDNA clone (pcA1; Sommer and Saedler, 1986). The results shown in Figure 1 indicate that CHS mRNA accumulated to high levels in the anterior and posterior regions of the flowers

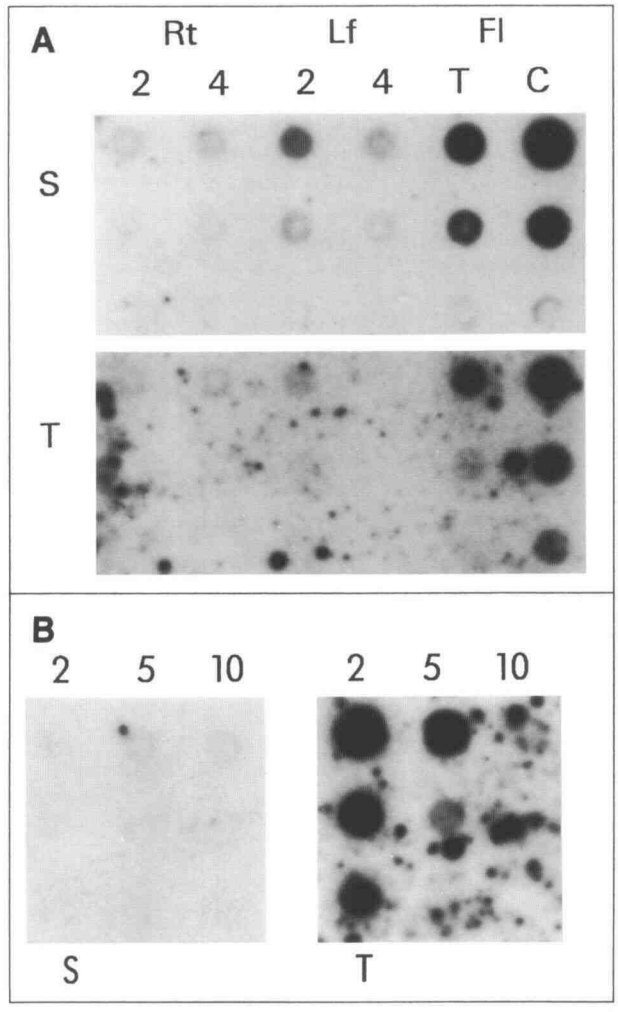

Figure 1. Accumulation of CHS mRNA in Different Tissues of Snapdragon and Tobacco.

(A) Total RNA isolated from root (Rt), leaf (Lf), and floral tissue (Fl) of snapdragon (S) and tobacco (T). RNA was isolated from either root and leaf tissue of 2- and 4-week-old plants, the floral tube (T), or corolla (C) of flowers.

(B) Total RNA isolated from maturing seeds of snapdragon (S) and tobacco (T) 2, 5, and 10 days after pollination. In both panels from top to bottom, 5, 1, and 0.2 μg of RNA were blotted on the filter and hybridized with radiolabeled pCS1 in tobacco and pcA1 in snapdragon. Subsequent hybridization of the blots with an actin clone served as an internal control for the levels of RNA used in each blot (data not shown).

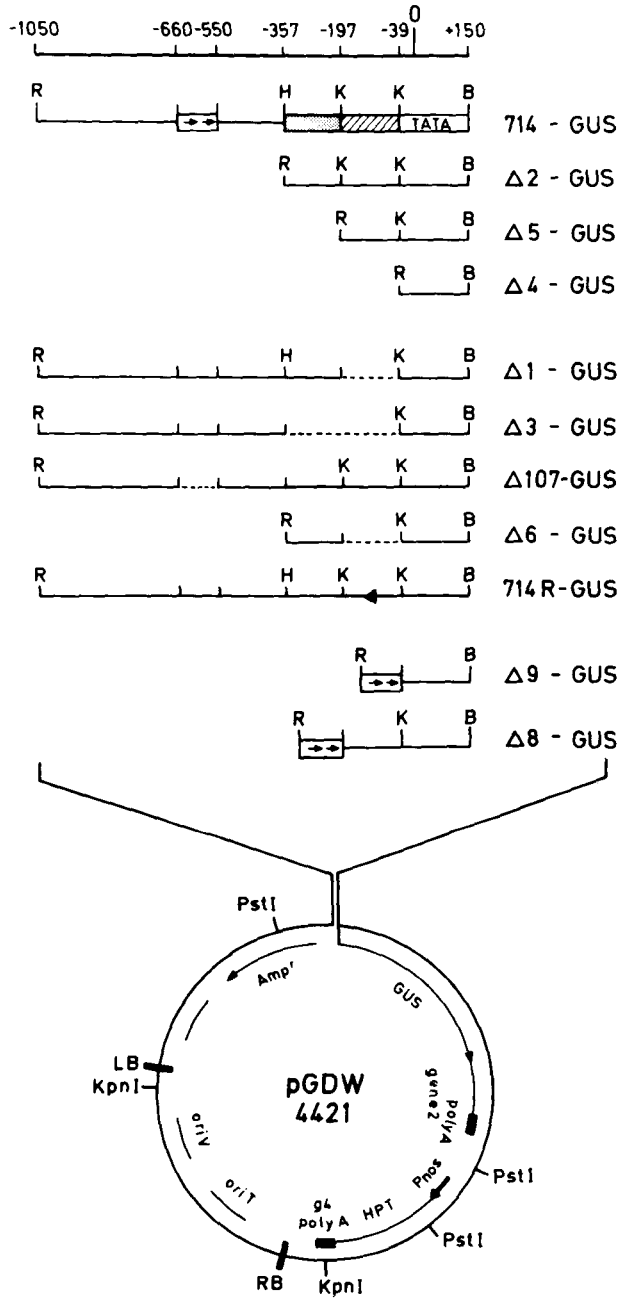

Figure 2. Chimeric CHS Promoter-GUS Fusions in the Vector pGDW4421.

Different regions of the 1.1-kb CHS promoter fragment were fused to the GUS structural gene linked to the polyadenylation site of gene 2 of the T-DNA of Agrobacterium. All constructs were subcloned into the binary vector pGDW4421 between the EcoRI (R) and BamHI (B) sites (H, HindIII; K, KpnI). In 714-GUS, the enhancer region between −660 and −550 is depicted as a box containing two arrows; the stippled box depicts the −357 to −197 region, which plays an accessory role in directing gene expression; the hatched box depicts the −197 to −39 region that contains the UV light-responsive element; the region containing the TATA box is indicated. GUS, β-glucuronidase gene; gene 2 poly(A), the polyadenylation signal from gene 2 of the T-DNA; g4 poly(A), the polyadenylation signal from gene 4 of the T-DNA; Pnos, the promoter of nopaline synthase gene; HPT, the hygromycin resistance gene; LB and RB, the left and right border sequences of the T-DNA; oriV and oriT, origins of replication functional in Agrobacterium; Amp^r, ampicillin resistance gene. Solid horizontal lines represent promoter sequences present in the clone. Broken lines represent internal deletions. The arrowhead in the 714R-GUS construct represents inversion of the −197 to −39 sequence.

of both plants. Maximum accumulation was observed in the corollae where pigmentation was most intense. This was not unexpected because of the role CHS plays in pigment formation (Harborne, 1976), and previous in situ hybridization results localized CHS-specific transcripts in tobacco flower tissue (Drews and Goldberg, 1988). In young leaf tissue, whereas low levels of CHS mRNA accumulation occurred in tobacco, higher levels were evident in snapdragon, possibly reflecting the requirement for CHS in the anthocyanin pigmentation of the abaxial surface of snapdragon leaves. Tobacco seeds accumulated high levels of CHS transcripts at the early stages of seed development (between 1 and 3 days postanthesis), whereas only trace amounts of CHS mRNA could be detected in snapdragon seed at similar stages of development. In roots of both plants, very little or no CHS mRNA accumulated at both stages of development tested.

Promoter-Reporter Gene Construction and the Production of Transgenic Plant Lines

DNA representing the 1.1-kb snapdragon CHS promoter and deletion derivatives thereof (Kaulen et al., 1986; Lipphardt et al., 1988) are shown in Figure 2. These were inserted as translational fusions with the GUS reporter gene in the binary plant transformation vector pGDW4421. Following transfer to Agrobacterium, these constructs were used to produce transgenic tobacco plants by leaf disc inoculation. Transgenic plants were selected for growth in the presence of hygromycin, and integration of the T-DNA containing the gene fusions was confirmed by DNA gel blot analysis, as well as by screening for GUS activity (see Methods). DNA gel blot analysis indicated that transgenic plants contained on average one to two copies of each construct per genome (data not shown). At least eight individual transformants representing each construct were tested for GUS activity. Plants were transferred to the greenhouse where an F_1 generation was obtained by selfing. When seeds of the F_1 generation of transgenic individuals were used, they were tested for the ability to germinate on hygromycin-containing media.

Table 1. Quantitation of GUS Activity in Different Tissues of Transgenic Plants Containing the GUS Gene Linked to the 1.1-kb Snapdragon CHS Promoter Fragment (714-GUS)

Tissue	GUS Activity (pmol methylumbelliferone/mg/min)
Leaf [a]	719
Leaf [b]	406
Stem[a]	947
Stem[b]	1,043
Root [a]	460
Root[b]	3,719
Callus[c]	12,978
Floral buds[d]	102
Petals, distal, pigmented[d]	5,748
Petals, posterior, white[d]	1,157
Ovary [d]	163
Seeds 3 to 6 days postfertilization[d]	14,573
Seeds 20 days postfertilization[d]	168

[a] Two-week-old F_1 generation seedlings from eight individual transformants tested.
[b] Four-week-old F_1 generation seedlings from eight individual transformants tested.
[c] Callus induced from stem sections of eight individual F_0 transformant seedlings.
[d] Material harvested from eight individual F_0 transformants.

The 1.1-kb Upstream Region of the Snapdragon CHS Gene Directs the Organ-Specific Expression of GUS in Transgenic Tobacco

Transgenic plants containing the 1.1-kb CHS upstream region fused to the GUS gene were dissected, and GUS activity was quantified in different organs by fluorometric assays. Moreover, to investigate possible developmentally regulated expression of the CHS-GUS chimeric gene, GUS activity was measured in leaf, stem, and root tissue samples taken from either 2- or 4-week-old seedlings from the F_1 generation. Table 1 presents the data obtained and demonstrates that the levels of organ-specific expression of the snapdragon CHS promoter in tobacco varied with the developmental stage. Figure 3 shows the localization of GUS activity in different organs revealed by histochemical staining. GUS activity was observed in most of the organs investigated, and the different levels of activity could be seen in different tissues, indicating that expression of the snapdragon CHS promoter in tobacco was controlled in both an organ-specific and tissue-specific manner.

The highest levels of GUS activity were observed in developing seeds shortly after fertilization. This result was expected because of the role of flavonoids in seed coat pigmentation (see Harborne, 1976) and correlates well with the observed accumulation of CHS mRNA of the endogenous tobacco genes (Figure 1). Developing seeds stained by 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) are shown in Figures 3D and 3E. Immature seeds from transgenic plants containing the chimeric GUS constructs were stained blue when they were dissected from fruits up to 1 week after anthesis. Seeds of the same stage of development from untransformed plants remain unstained. Only low levels of GUS expression were detected in the ovaries from open flowers before pollination, with peak GUS activity being seen as soon as the developing seeds could be isolated.

In the mature flowers of transgenic plants, GUS activity was approximately 5 times higher in the distal, pigmented region of the petal than the basal, white region (Table 1). Figures 3B and 3C show that this observation is confirmed by the histochemical staining of mature flower petals with X-gluc. Cells from different tissues in the pink-pigmented distal part of the petal are stained blue by X-gluc, i.e., epidermal, vascular tissue, and trichomes. These observations correlate well with the patterns of accumulation of the endogenous CHS transcripts in both tobacco and snapdragon (Figure 1) and indicate that the snapdragon CHS promoter is functioning normally in a tissue-specific manner in petals of transgenic tobacco. Microscopic analysis of GUS expression in flower tissue revealed that there is no distinct demarcation of cells expressing or not expressing GUS activity at the pink/white border; rather, there is a gradual change in the levels of expression (Figure 3C). This would suggest that either expression may be controlled by diffusable rather than cell-specific factors or that there is no strict border between different cell types.

In vegetative tissue, the highest GUS activities were observed in root tissue (Table 1). There is an apparent ninefold higher level in GUS activity in 4-week-old roots when compared with 2-week-old roots. Histochemical

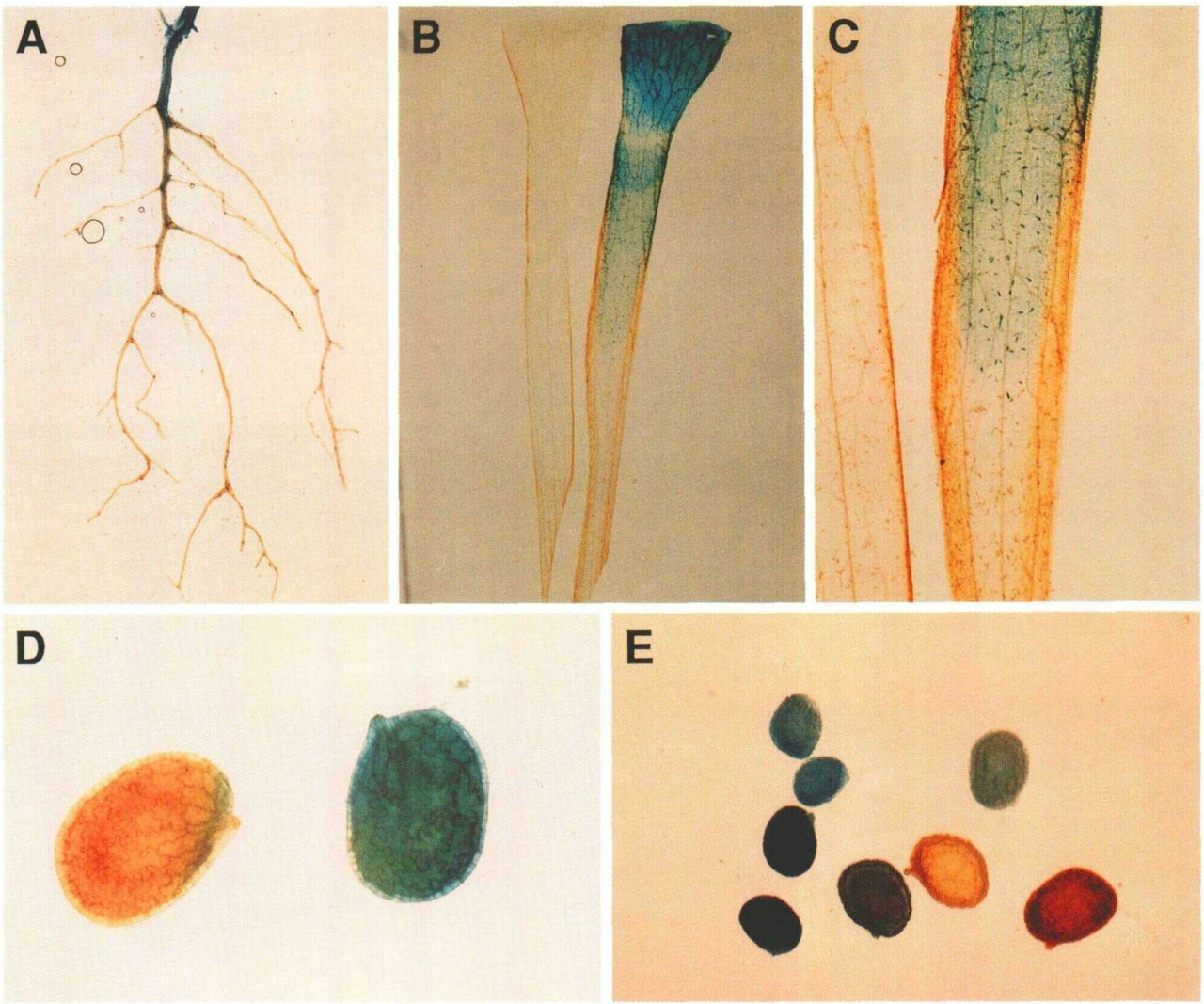

Figure 3. Histochemical Localization of GUS Expression in Transgenic Tobacco Plants Containing the 1.1-kb CHS Promoter Linked to GUS.

(A) Roots from a 4-week-old-seedling.
(B) Petal tissue from untransformed (left) and transformed (right) plants.
(C) Magnification of petal from **(B)**.
(D) Seeds dissected 5 days after self-pollination from a seedpod of untransformed (left) and transformed (right) tobacco plant.
(E) Seeds dissected from seedpods in different developmental stages (3 to 20 days after pollination) from a transformed tobacco plant.
In each case, transformed plant refers to a plant containing the 1.1-kb CHS promoter fragment linked to GUS.

staining showed that GUS activity in roots driven by the CHS promoter is localized in the cells of the older parts of the root, primarily the epidermal cells (Figure 3A). This is in contrast to the observations of the accumulation of CHS transcripts from the endogenous genes in both tobacco and snapdragon shown in Figure 1, where little or no accumulation of CHS mRNA could be detected. This could imply that either CHS expression is posttranscriptionally regulated in roots or that the 1.1-kb promoter region lacks a root-specific silencing activity. The levels of GUS activity in 2-week-old leaves are slightly higher than that in 4-week-old leaves, and this correlates with the levels of CHS transcripts accumulating in both tobacco and snapdragon leaves. However, the absolute level of GUS activity is

relatively low in transgenic tobacco leaf tissue, reflecting the lower accumulation of CHS mRNA in tobacco rather than that seen in snapdragon leaves. GUS activities in 2- and 4-week-old stem tissue are essentially the same.

When callus growth was induced from stem tissue, the resulting callus routinely displayed high levels of GUS activity (Table 1). Considering the organ-specific and tissue-specific expression of the promoter, it is possible that in callus expression has become deregulated or that the conditions of tissue culture have induced expression of the promoter.

Deletion Derivatives of the Snapdragon CHS Promoter Direct Different Patterns of Expression in Transgenic Tobacco

The analysis of transgenic plants containing the 1.1-kb snapdragon CHS promoter-GUS fusion showed a finely orchestrated system of promoter control involving organ-specific and tissue-specific as well as developmental regulation. Hence, to define regions of the promoter necessary for the differential expression of the CHS promoter, GUS expression directed by deletion derivatives of the promoter fused to the GUS reporter gene, shown in Figure 2, were analyzed in different organs of transgenic tobacco plants.

Sequences up to −39 (Δ4-GUS) of the CHS promoter were sufficient to direct gene expression in root and stem tissue, as shown in Figure 4. Plants containing Δ4-GUS displayed an increase in GUS expression in roots between 2 and 4 weeks of age. However, if sequences up to −197 (Δ5-GUS) were present, no GUS activity could be detected in roots, suggesting that the region −197 to −39 contains an element that has a negative effect on expression in roots. Because sequences up to −357 (Δ2-GUS) conferred higher levels of expression in roots, it would appear that the effect of this negative element can be overridden by upstream elements located between −357 and −197. The level of expression directed by Δ2-GUS appears to be approximately half of that of the intact promoter. An internal deletion of the region containing the 47-bp repeat located between −674 and −564 (Δ107-GUS) reduced the activity of the promoter when compared with Δ2-GUS. This suggests that this region is largely responsible for maximal levels of expression conferred by the region between −1050 and −357. Moreover, it shows that sequences apart from the 47-bp repeat within the −1050 to −357 region have a negative effect on expression. Fusion of the sequence containing the 47-bp direct repeat at position −39 (Δ9-GUS) increased the levels of GUS activity in 2-week-old tissue. In addition, the repeat sequences appear to be able to overcome the effect of negative elements localized between −197 and −39 (Δ8-GUS). However, in Δ9-GUS and Δ8-GUS, there appears to be no increase in GUS activity between 2- and 4-week-old tissue, suggesting that these constructs lack sequences for developmental regulation of expression in the transgenic system. The influence of 5′ upstream regions on expression in roots directed by the TATA-proximal region is demonstrated further by the construct 714R-GUS. Here, placement of the region between −197 to −39 in a reverse orientation results in a decrease of expression in roots.

The minimal sequences of the CHS promoter necessary for expression in leaf tissue can be delimited to the region

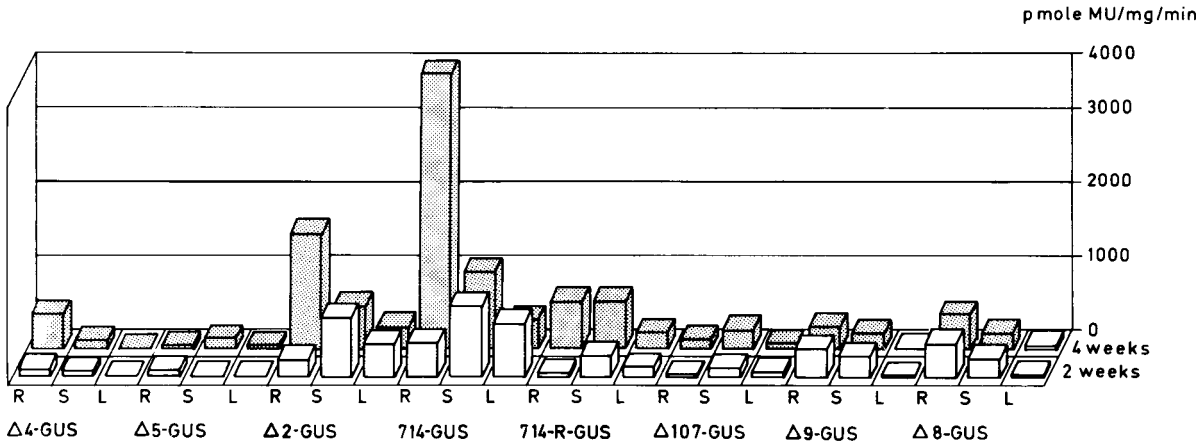

Figure 4. Quantitation of GUS Activity in Different Tissues of Transgenic Seedlings at Differing Developmental Stages.

Each set of bars of the histogram represents GUS activity in root (R), stem (S), or leaf (L) tissue of either 2- or 4-week-old seedlings of F_1 plants containing the indicated construct. Extracts were standardized and GUS activity was assayed by fluorimetry as described in Methods. MU, 4-methylumbelliferone.

extending to −357 (Δ2-GUS). This assumption is based on the observation that no GUS activity is observed in leaf tissue of transgenic plants containing Δ4-, Δ5-, Δ9-, and Δ8-GUS (Figure 4). Comparison of the results obtained with Δ2-GUS and Δ107-GUS in leaf tissue indicates that in a manner similar to that in roots, the 47-bp repeat sequence located between −674 and −564 is a major contributor to the levels of expression observed and that other regions between −1050 and −357 have a negative effect on expression. Moreover, reversal of the orientation of the sequences located between −197 and −39 (714R-GUS) results in a decrease in expression in leaf tissue when compared with the unrearranged 1.1-kb region (714-GUS). Internal deletion of the −197 to −39 region (Δ1-, Δ6-, and Δ3-GUS) results in a complete loss of GUS activity not only in leaf tissue but also in roots and stems (data not shown). This suggests that sequences from −357 to −197 and −197 to −39 are required in their normal position for CHS expression in leaves.

As shown in Figure 5A, expression of the snapdragon CHS promoter in tobacco appears to be highly regulated during seed development, with maximal GUS activity being observed for 3 days, after which there is a rapid decline. Hence, to investigate the seed-specific expression of the deletion derivatives of the promoter, seeds were examined 4 to 6 days postanthesis because it was found that with the 1.1-kb promoter fragment there is a plateau in GUS activity at this stage of development. Figure 5 shows that the minimal sequences required for seed-specific expression are located between −197 and −39 (Δ5-GUS). In contrast to root, stem, and leaf tissue, the enhancer sequence located between −674 and −564 does not make the largest contribution to the levels of expression obtained. Its deletion reduces the level of expression by 50% compared with the intact 1.1-kb fragment. Sequences between −357 and −197 also appear to contribute to produce maximal expression in seeds. Moreover, the −197 to −39 region appears also to have a negative effect on the ability of the 47-bp repeat region to increase levels of expression (compare Δ4-GUS and Δ9-GUS with Δ5-GUS and Δ8-GUS). In contrast to vegetative tissue, reversal of the −197 to −39 sequence has no effect on the levels of expression seen in seeds. Hence, the seed-specific regulation of the promoter constructs is different from that seen in petals (see below) and vegetative tissues, suggesting an independent system of factors or elements controlling expression in seed.

Figure 6 shows the levels of GUS expression observed in pink and white portions of the flower controlled by different promoter constructs. In each of the constructs tested, between a twofold and fivefold higher level of expression was found in the pigmented region of the petal. As in seeds, the region −197 to −39 also directed GUS expression in petals, with a twofold greater effect in the pigmented sectors. Inversion of the −197 to −39 region within the 1.1-kb fragment (714R-GUS) had little effect on

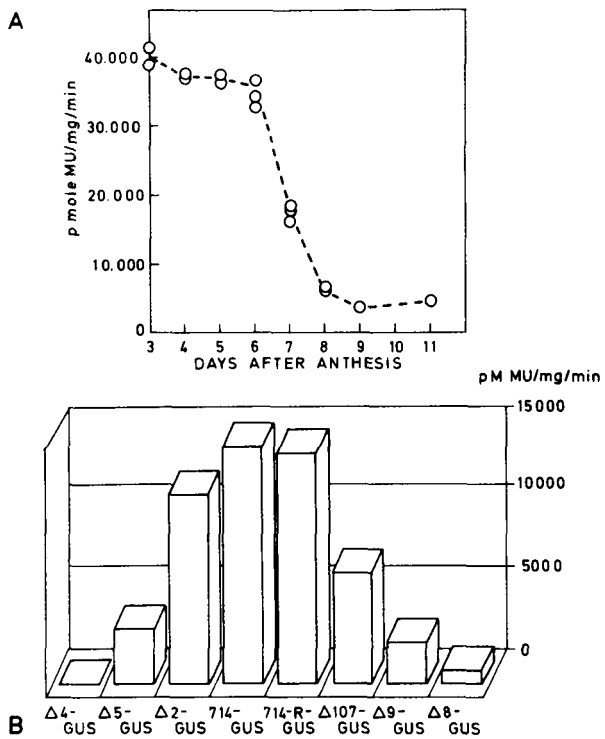

Figure 5. Quantitation of GUS Activity in Developing Seeds from Transgenic Plants.

(A) Time course of appearance of GUS activity in extracts of developing seeds dissected from immature seedpods from a transgenic individual containing the full CHS promoter fused to the GUS gene.

(B) GUS activity in seeds isolated 3 to 6 days postanthesis from transgenic plants containing different regions of the CHS promoter fused to the GUS gene. Extracts were prepared and standardized as described in Methods. MU, 4-methylumbelliferone.

petal-specific GUS expression, indicating its function independent of orientation, whereas its deletion (Δ1-GUS, Δ6-GUS) abolished all activity (data not shown). Maximal expression in pigmented sectors of petals was conferred by sequences 5′ to this region, as seen by sequential addition of the −357 to −197 (Δ2-GUS) and the −1050 to −357 (714-GUS) regions. However, in contrast to expression seen in other tissues, deletion of the enhancer sequence located between −674 and −564 does not reduce the levels of GUS activity in petals; indeed, it is slightly increased. This is true in both the pink posterior region and the white anterior region of the petal and suggests that the sequences located between −674 and −564 do not contribute to the enhancing effect provided by the −1050 to −357 region in petals. This finding is supported by the observation that fusion of the region containing the 47-bp repeat sequence to the promoter deleted to −197

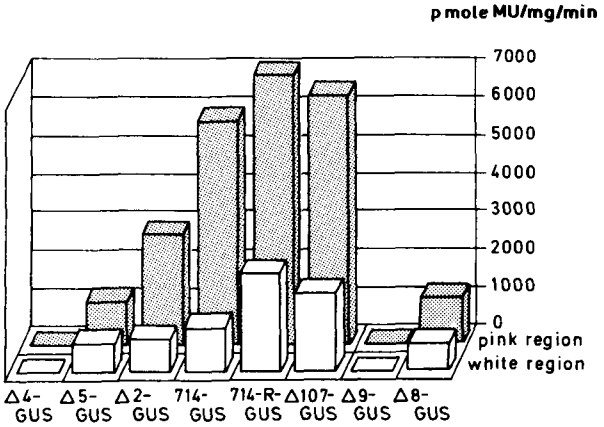

Figure 6. Quantitation of GUS Activity in Petals of Transgenic Plants.

Each set of bars of the histogram represents GUS activity in the posterior pink-pigmented or anterior white-pigmented part of the petal. Extracts were standardized and assayed as described in Methods. MU, 4-methylumbelliferone.

and −39 (Δ8-GUS and Δ9-GUS, respectively) does not alter the levels of GUS expression, in contrast to that seen in roots and seeds. Hence, it would appear that the sequences located between −674 and −564 could be considered as an organ-specific enhancer directing maximal expression in leaf, stem, roots, and developing seeds but not in petals. In petals, it would appear that other regions located between −1050 and −357 are responsible for maximal expression of the promoter.

Chimeric CHS-GUS Genes Can Be Induced by UV Light in Transgenic Tobacco Seedlings

The synthesis of phenylpropanoids has been implicated as being a plant defense response to UV light (for review, see Dangl et al., 1989). Previously, it has been shown that the 1.1-kb snapdragon CHS promoter fragment linked to the NPTII gene is UV light inducible in tobacco seedlings (Kaulen et al., 1986). Hence, we were particularly interested in analyzing the expression of the CHS promoter deletions in transgenic plants after UV light induction. To do this, seeds from the F_1 generation of eight individual transformants were sown and grown under plexiglass shields to protect them from UV light. UV light induction was carried out by exposing the seedlings to continuous UV light (see Methods). Leaf tissue of the seedlings was harvested at different times after the onset of irradiation and assayed for GUS activity. As shown in Figure 7, with the full promoter we observed an increase in expression in leaf tissue with an approximate 5.5-fold increase in GUS activity occurring after 48 hr irradiation by UV light. Parallel experiments indicated that under white light conditions no induction of GUS activity was found during the time course of the experiment (data not shown). The induction of the 1.1-kb CHS promoter fragment in transgenic tobacco seedlings upon exposure to UV light has been shown to display the same kinetics as an endogenous tobacco CHS gene, as judged by RNA gel blot analysis (D. Staiger unpublished data). When the region spanning −1050 to −357 (Δ2-GUS) is deleted, there is a reduction in the level of expression (approximately twofold), although UV light induction is retained. Deletion of the 47-bp direct repeat element (Δ107-GUS) reduces the expression in induced seedlings approximately 75%; however, the kinetics of induction obtained with Δ107-GUS are the same as the complete promoter, demonstrating that the region −674 to −564 acts as an enhancer in UV induction and apparently has no direct involvement in the perception of UV light. A similar result is obtained when the region −197 to −39 is inverted, indicating that this element is either not important in UV induction or it functions in an orientation-independent manner.

Further deletion of the promoter to −197 and −39 (Δ4-GUS and Δ5-GUS) abolished detectable promoter activity in leaf tissue (Figure 4) and in this case also no UV light inducibility was observed (Figure 7). Expression of these

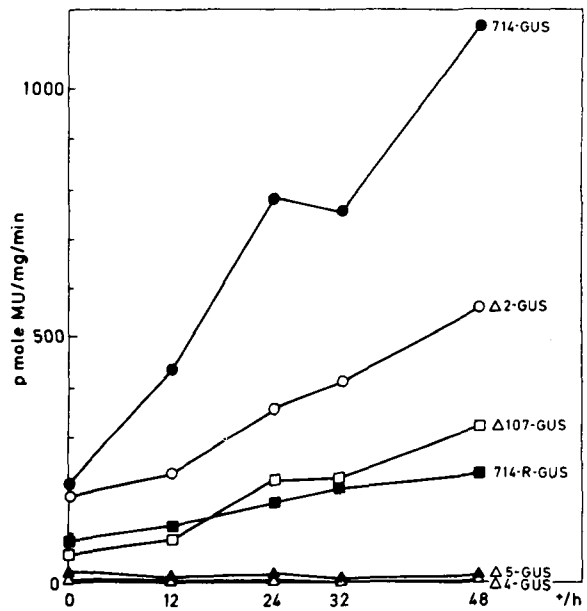

Figure 7. Quantitation of GUS Activity in Leaves from 6-Week-Old Seedlings Exposed to UV Light.

Time course of appearance of GUS activity in extracts from leaves of tobacco seedlings continuously irradiated with UV light. Irradiation and assays were performed as described in Methods.

constructs in other tissues suggests that sequences essential for transcription in general have not been removed, but rather those responsible for expression in leaves in normal conditions as well as after UV light induction.

DISCUSSION

Recently, using CHS promoters derived from either French bean or petunia fused to marker genes, it has been found that expression can be tightly regulated in both a tissue-specific and developmentally regulated manner (Schmid et al., 1990; Koes et al., 1990). Because the promoters used in these studies were single members of small, multigene families, it was of particular interest to see whether the single snapdragon CHS promoter was regulated in a similar manner. Until now, the *cis* analysis of the snapdragon CHS promoter has been carried out either in a callus cell line (Kaulen et al., 1986) or by transient expression in protoplasts (Lipphardt et al., 1988). These systems were able to define two types of regulatory sequence: those that are important in directing high levels of gene expression and those conferring UV light induction. Within the snapdragon CHS promoter, the region −661 to −564 appeared to act as a general enhancer, whereas the region −357 to −197 acted to enhance levels of UV light-induced gene expression when linked to the UV light-responsive element located −197 to −39 (Lipphardt et al., 1988). However, by their very nature, these experimental systems were unable to address the question of whether specific sequences are important in directing tissue-specific or developmentally regulated expression.

Currently, there is no transformation protocol for snapdragon. However, previously it had been demonstrated that the 1.1-kb upstream region of the snapdragon CHS gene confers a high level of expression on a linked marker gene in transgenic tobacco callus and UV light-induced seedlings (Kaulen et al., 1986). Hence, we chose transgenic tobacco as an experimental system to carry out a functional analysis of the CHS promoter to delineate those sequence elements directing organ-specific and developmentally regulated expression. In this paper, we demonstrate that a 1.1-kb fragment of the snapdragon CHS promoter can function differentially in transgenic tobacco and, hence, deletion derivatives of the promoter can be used to determine those regions of the promoter directing organ-specific and developmentally regulated expression in tobacco. The data presented in this paper extend our knowledge of the functional organization of the CHS promoter obtained by transient assays, and we have been able to define at least five functional regions of the promoter.

We show that as short a region as that extending to −39 and containing the TATA box is sufficient to direct expression in root and stem tissue. Such a small region of a promoter conferring a specific pattern of expression is not without precedent. For example, it has been shown that a 33-bp sequence contained within the promoter of a pea gene encoding ribulose-bisphosphate carboxylase is involved in the light inducibility of the gene (Morelli et al., 1985).

The region extending from −197 to −39 appears to have at least three attributes. First, it contains sequences necessary for expression of chimeric constructs in petals and developing seeds. This is the same region previously found to be important for CHS expression in petals in snapdragon as a result of transposon insertion and deletion (Sommer et al., 1988). It is striking that the levels of accumulation of the endogenous CHS mRNA in the developing seeds of snapdragon and tobacco differ. Expression of the snapdragon promoter in tobacco suggests that it follows the expression pattern of the endogenous tobacco promoter during seed development. This suggests that it is responding to endogenous tobacco factors whose counterparts in snapdragon seeds are either lacking or serve to produce lower levels of expression. The second attribute of the −197 to −39 region is that in root tissues it appears to have a negative effect on expression directed by sequences which flank its 3′ end. Finally, in the leaf, although the −197 to −39 region alone is ineffective in directing tissue specificity, it is required for expression. The notion that the −197 to −39 region is important in CHS expression is supported by the finding within this region of a CACGTG motif that has been demonstrated recently to bind a factor, CG-1, present in nuclear extracts (Staiger et al., 1989).

The 160-bp fragment extending from −357 to −197 acts as a positive regulatory element in CHS promoter expression in all the tissues investigated. In petals and developing seeds, these sequences act in concert with the −197 to −39 region to direct a higher level of expression than that of the −197 to −39 region alone. In leaves, roots, and stems, we observe almost no expression in the absence of the −357 to −197 region. Thus, we conclude that a combination of this region with the −197 to −39 sequence is necessary to direct expression. This would indicate an interplay between recognition sites for factors that activate transcription located on both fragments. This idea is supported by the finding that reversal of the −197 to −39 region results in a severe decrease in expression in leaves, stems, and roots. The −357 to −197 fragment does not play the role of an enhancer in the generally accepted sense because fusion of this sequence to the promoter truncated to −39 (Δ6-GUS) does not increase expression. Indeed, in this case, no expression is observed (data not shown). Rather, the 160-bp sequence works in concert with the −197 to −39 to potentiate expression.

The −357 to −197 region appears to be essential for leaf-specific expression, and deletion of this region abolished UV light induction in leaf tissue. In contrast, constructs containing only the −197 to −39 fragment are

sufficient for UV light induction in protoplasts (Lipphardt et al., 1988). This suggests that in leaf tissue the UV light-responsive element in itself is not sufficient to ensure expression and regions located between −357 to −197 are required. This requirement is more specific than simple enhancement because linking the cauliflower mosaic virus 35S RNA enhancer (defined as −392 to −90; Franck et al., 1980) to the −197 to −39 region does not result in UV light inducibility in leaf tissue (K. Fritze, unpublished data). Taken together, this suggests that UV light induction and sequences important in directing expression in leaf tissue work cooperatively in the intact promoter. Two mechanisms for this cooperation in leaf tissue could be envisaged. One might be that factors important in expression in leaf tissue cooperate with UV light-induced factors that recognize separate promoter elements. A similar situation has been described for the promoter of the small subunit of ribulose-bisphosphate carboxylase where upstream tissue-specific regulatory sequences are required for the function of downstream light-responsive elements (Kuhlemeier et al., 1988). On the other hand, factors important in expression of CHS in leaf tissue might respond to a specific signaling factor and bind more frequently or more tightly to mediate the further increases in CHS promoter activity. Both models conform to the results of the analysis of the parsley CHS promoter, which identifies two UV light-induced in vivo footprints between −275 and −100 (Schulze-Lefert et al., 1989b) where the observed "boxes" would still be preferentially protected upon UV irradiation.

The region −1050 to −357 contains elements essential for maximal expression of the promoter (Kaulen et al., 1986; Lipphardt et al., 1988), and we have delineated the sequences responsible to this effect as being between −674 to −564. That this sequence acts as an enhancer and that the decrease in expression observed upon its removal does not result simply from the change of spacing of elements on either side of it is suggested by two observations. First, the 47-bp direct repeat can be placed 5′ of other sequence elements and enhance expression. Second, the sequence of the repeat resembles that of other enhancers (Kaulen et al., 1986). The 47-bp direct repeat sequence itself has been found to contain three binding sites for a nuclear protein factor from tobacco and snapdragon (Staiger et al., 1990). We have found that the enhancer-like function acts specifically in root, stem, leaf, and immature seeds. Moreover, this region acts as an enhancer directing high levels of CHS expression after UV light induction. In contrast, this region does not appear to be active in petals, indicating that the enhancer effect operates in a tissue-specific manner. In petals, sequences other than those located between −674 to −564 in the region −1050 to −357 act to produce maximal expression from the CHS promoter. Indeed, the sequence −1050 to −357, when lacking the 47-bp direct repeat, appears to act differently in different tissues. In addition to the enhancer-like effect revealed in petals, a silencing effect is observed in immature seeds, stem, and roots. These subtle effects are only manifested upon removal of the 47-bp direct repeats, the upstream enhancer appearing to have a dominant effect on the pattern of expression. This dominant effect, at least in the case of UV light induction, cannot be replaced by the enhancer derived from the cauliflower mosaic virus 35S RNA promoter; although the general levels of expression are increased, UV light induction is abolished (K. Fritze, unpublished data).

During the course of this work, we gained some insight into the limitations of using GUS assays to analyze promoter deletions in transgenic plants. GUS as a marker gene has well proven itself to be a versatile assayable marker, but as discussed elsewhere (for example, see Benfey et al., 1989), there are several inherent difficulties in comparing the resulting data. As yet, there is no marker that allows quantitation of expression on a per-cell basis; hence, in tissue comprising different cell types, quantitation on a per-cell basis can be difficult. We have attempted to overcome this problem by presenting data on a per-microgram-of-protein basis, and the results obtained have compared well with observations made with histochemical staining (K. Fritze, unpublished data). In an attempt to circumvent possible position effects, at least eight individuals for each construct were measured and the data averaged. In the cases where this measurement was unexpectedly high or low (for example, the expression observed in the roots of Δ4 in 4-week-old seedlings), individual samples from the eight plants were taken and measured separately to ensure that the average increase/decrease was not due to an aberrant individual. Throughout, the variance in the levels of GUS expression was always within 20% of the average value.

It has become increasingly clear that of those plant promoters studied in detail, their general organization is such that sequences important in directing tissue-specific or developmentally regulated expression are located near the TATA box, and elements directing high levels of expression are located 5′ to these regions (Simpson et al., 1986; Chen et al., 1988; Poulsen and Chua 1988). The snapdragon CHS promoter conforms to this pattern of organization. In this respect, it is similar to the parsley CHS promoter, where sequences important in directing high levels of expression are positioned 5′ to those involved in the response to UV light (Schultze-Lefert et al., 1989a). In this case, the enhancer sequences are located between −534 and −463, whereas two light-responsive elements characterized by two light-induced in vivo footprints are located between −275 and −100 (Schulze-Lefert et al., 1989b).

Our analysis of the snapdragon CHS promoter suggests that it comprises a collection of sequence elements, which

may or may not overlap, interacting to direct the characteristic expression of the gene. We have shown that different regions of the promoter are important in directing the expression of the CHS gene in different tissues at different stages of development. Moreover, we have found that sequence motifs within the promoter can interact differentially in either a positive or negative manner in directing expression. In some tissues, a sequence may act as an enhancer overriding the effect of sequence elements active at another stage of development. Hence, the CHS promoter may be viewed as a complex array of sequence motifs, each of which defines a specific pattern of expression that in the entire 1.1-kb promoter fragment interact to produce a characteristic pattern of expression. This pattern of expression is likely to be controlled by the binding characteristics as well as the availability in different cell types and tissues of the different *trans*-acting factors that interact with the *cis* regulatory sequences contained within the promoter.

METHODS

Materials

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs or Boehringer-Mannheim Biochemicals and used according to the suppliers' instructions. Other chemicals were obtained from Aldrich or Sigma.

Bacterial Strains and Plasmids

Escherichia coli K12 MC 1061 (Casadaban and Cohen, 1980) was used throughout for the different cloning steps. For Agrobacterium-mediated plant transformation, the binary vector pGDW4421 was used. pGDW4421 was derived from pGDW44 (Wing et al., 1989), and the salient features of it are a hygromycin resistance gene that is functional in plant cells and a promoterless GUS reporter gene located between the T-DNA border sequences (Figure 1). Different promoter constructs cloned into pGDW4421 were transferred to *Agrobacterium tumefaciens* pGV3101 (Van Larebeke et al., 1974) harboring the Ti plasmid pMP 90 RK (Koncz and Schell, 1986) by conjugation with *E. coli* Sm10 S17.1 (Simon et al., 1983) containing each of the individual constructs. Exconjugants were selected by antibiotic resistance (Draper et al., 1988) and screened by minipreparations of plasmid DNA (Ebert et al., 1987) or "back-conjugation" (Koncz and Schell, 1986).

Recombinant DNA Techniques

DNA isolation, modification, bacterial transformation, and clone identification were performed using standard procedures (Maniatis et al., 1982). Regions of the *Antirrhinum majus* promoter were subcloned as EcoRI-BamHI fragments from the different promotor constructions described previously (Kaulen et al., 1986; Lipphardt et al., 1988, Figure 2). To construct Δ107-GUS, an internal deletion between −674 and −564 was made by cutting with HpaII followed by Bal31 digestion of the 1.1-kb CHS promoter. The sequence from −1050 to −674 was inserted between the EcoRI and SmaI sites of pUC19. This plasmid was cut with HincII, and the sequence −564 to −357 region of the wild-type promoter was inserted at this site as a blunt-ended HpaII and HincII fragment. From the resultant plasmid, an EcoRI-SnaBI fragment spanning the −1050 to −400 region of the mutated promoter was transferred to 714 pUC (Lipphardt et al., 1988). The area of the deletion was confirmed by sequencing. Promoter fragments, linked to the first 18 codons of the CHS gene, were ligated to the GUS reporter gene to yield a translational fusion in pGDW4421.

Dot Blot Analysis

RNA was isolated using a guanidinium thiocyanate extraction protocol and purified through CsCl step gradients (Chirgwin et al., 1979). Amounts of RNA were estimated spectrophotometrically. The 0.2, 1, and 5 μg of RNA were dotted onto nylon membranes and hybridized in the presence of 50% formamide and washed using standard procedures (Maniatis et al., 1982). Hybridization was visualized by autoradiography. Hybridization of the filter with an actin clone (pSAC1, kindly provided by Richard Meagher, University of Georgia, Athens) was used to confirm correct quantitation of the RNA bound to the filter (data not shown).

Plant Transformation

Transformation of *Nicotiana tabacum* cv Wisconsin 38 was performed using the leaf disc procedure described by Horsch et al. (1985). After inoculation with *A. tumefaciens* harboring the various promoter constructs, the leaf tissue was cultured on LS medium (Linsmaier and Skoog, 1965) supplemented with hygromycin (15 mg/L), cefotaxime (500 mg/L), naphthaleneacetic acid (0.1 mg/L), and benzylaminopurine (0.5 mg/L). Shoots growing on this medium were transferred to LS medium containing the same antibiotics but no hormones to allow rooting. Hygromycin-resistant plants were transferred from axenic culture to the greenhouse, where F_1 plants were produced by selfing. DNA gel blot analysis was carried out as described previously (Maniatis et al., 1982; Wing et al., 1989).

Assaying for GUS Activity

Preparations of crude plant extracts were assayed as described by Jefferson et al. (1987), with extracts standardized by assessing protein concentration using the method of Bradford (1976). GUS levels in different plant tissues were assayed by enzymatic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone, which was quantified with a fluorimeter (Perkin-Elmer LS 2B) set at 455 nm emission and 365 nm excitation wavelengths. Throughout, GUS activity is expressed as picomoles per milligram of protein per minute.

Histochemical staining of transgenic tissue used X-gluc, as described previously (Jefferson et al., 1987). Stained tissues were rinsed with ethanol and stored in glycerol.

In comparison with the GUS activity in leaves, stem, and roots from the F_1 generation, equal amounts of seeds from eight individual transformants containing each construct were mixed and sown in soil. Two and 4 weeks after germination, seedlings were harvested and dissected into roots, leaves, and stems and assayed for GUS activity. For the analysis of GUS expression in flowers, four petals of open flowers from eight individual transformants containing one of the constructs were dissected. In each case, pink-pigmented and white tissue were analyzed for GUS expression by fluorimetric assays. To analyze GUS expression in developing seed, seedpods of the transgenic tobacco plants were collected 3 to 6 days after fertilization, and the seeds were dissected and assayed for GUS activity. Stem callus from transgenic tissue was induced by culturing axenic stem sections on LS medium supplemented with naphthaleneacetic acid (1 mg/L) and benzylaminopurine (0.2 mg/L).

UV Light Induction of Transgenic Seedlings

Equal amounts of seed from the offspring of eight individual transformants containing each construction were mixed and sown in earth. Seedlings were grown in a greenhouse protected from UV light by plexiglass shields. Six-week-old seedlings were irradiated by exposure to UV light at the onset of the photoperiod, as previously described by Kaulen et al. (1986). Leaf tissue was harvested from irradiated plants 0, 12, 24, 32, and 48 hr after treatment, frozen in liquid nitrogen, and analyzed by fluorimetric GUS assays as described above.

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