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Pollen- and Anther-Specific *chi* Promoters from Petunia: Tandem Promoter Regulation of the *chi*A Gene

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We have analyzed the spatial and temporal activities of chalcone flavanone isomerase (*chi*) A and B gene promoters from petunia. To study the tandem promoter regulation of *chi*A, various *chi*A promoter fragments were fused with the β-glucuronidase (GUS) reporter gene. Analysis of transgenic plants containing these chimeric genes provided definitive proof that the *chi*A coding region is regulated by two distinct promoters (designated P_{A1} and P_{A2}). We also showed that both promoters can function independently and that the *chi*A P_{A1} promoter is expressed in limb (epidermal and parenchyma cells), tube (inner epidermal and parenchyma cells), seed (seed coat, endosperm, and embryo), sepal, leaf, and stem. The use of *chi*A and *chi*B promoters in the regulation of anther- and pollen-specific gene expression has been studied. By analyzing transgenic plants containing chimeric genes consisting of *chi*A and B promoter fragments and the GUS reporter gene, we were able to identify a 0.44-kilobase *chi*A P_{A2} promoter fragment that drives pollen-specific gene expression and a 1.75-kilobase *chi*B P_B promoter fragment that confers anther-specific (pollen and tapetum cells) expression to the GUS gene.

INTRODUCTION

Flower development is a complicated process in which organs such as sepals, petals, anthers, and carpels are formed that develop complex interrelationships (for reviews, see Goldberg, 1988; Drews and Goldberg, 1989). Recent work by Weiss and Halevy (1989) indicates that anthers are essential for flower development and are thought to be involved in the production of gibberellins necessary for the growth and pigmentation of petunia flowers (Weiss and Halevy, 1989). Our goal is to understand how development of the flower is regulated and coordinated at the molecular level.

The generation of flower color through the accumulation of flavonoids is an ideal marker of flower development. Flavonoids, especially the anthocyanins, are the main flower pigments and form much of the yellow, red, orange, and purple colors in floral tissues (van Tunen and Mol, 1989). Flavonoid biosynthesis is an excellent system to study because a wealth of fundamental knowledge is available, and many genes encoding the various flavonoid biosynthesis enzymes have been isolated (Mol et al., 1988). In petunia, genes encoding chalcone synthase (CHS), chalcone flavanone isomerase (CHI), and dihydroflavonol reductase (DFR) have been cloned and characterized (van Tunen et al., 1988; Beld et al., 1989; Koes et al., 1989a, 1989b; van Tunen et al., 1989). Members of the CHS, CHI, and DFR multigene family are coordinately expressed in corollas and anthers during flower development (van Tunen et al., 1988; Beld et al., 1989).

CHS is the first enzyme of flavonoid biosynthesis and converts malonyl CoA and 4-coumaroyl-CoA into narigenin chalcone. CHI, the consecutive enzyme, isomerizes the chalcone into the corresponding flavanone. In petunia CHI is encoded by two genes, *chi*A and *chi*B, that have been characterized previously (van Tunen et al., 1988, 1989). *chi*A contains no intervening sequences, whereas the *chi*B coding region is interrupted by three introns. A combination of RNase protection experiments and primer extension analyses strongly indicated that *chi*A is regulated by two distinct promoters that act in tandem and are differentially used; the downstream P_{A1} promoter is active in the petals (giving rise to a 1.0-kb mRNA), whereas the upstream P_{A2} promoter is active in mature anthers and, more precisely, in pollen grains (giving rise to a 1.5-kb RNA transcript). The *chi*B gene, on the other hand, is regulated by a single promoter, P_B, which is active in immature anther tissue (resulting in the accumulation of a 1.0-kb transcript). In a previous paper (van Tunen et al., 1989), we hypothesized that a highly conserved region in the P_B promoter, designated as the "antherbox," plays a regulatory role in anther-specific gene expression.

The molecular basis of regulatory processes controlling pollen and anther development is only poorly understood (for review, see Mascarenhas, 1989). To gain insight into the regulation of anther- and pollen-specific gene expres-

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sion, several laboratories have carried out differential screening of cDNA libraries and have obtained cDNA clones corresponding to mRNAs expressed in various cell types of tobacco or tomato anthers (Goldberg, 1988; Ursin et al., 1989) and in pollen from maize, *Tradescantia paludosa*, and *Oenothera organensis* (Stinson et al., 1987; Hanson et al., 1989; Mascarenhas, 1989). Until now only a few anther-specific genes have been characterized. The *Lat52* gene was isolated from tomato and encodes an 800-nucleotide-long transcript accumulating in pollen, anthers, and, at 20-fold to 50-fold lower levels, in petals (Twell et al., 1989). The maize *Zmc13* encodes a 1.0-kb mRNA that accumulates only in the mature pollen and more specifically in the cytoplasm of the vegetative cell (Hanson et al., 1989). However, the precise regulatory sequences of these genes have not yet been identified. In this paper we report a detailed analysis of various *chi* promoter regions active at early and late stages of anther development. Various DNA fragments of pollen- and anther-specific *chi* promoters were isolated and fused in front of the β-glucuronidase (GUS) reporter gene (Jefferson et al., 1987). Subsequently, transgenic plants were analyzed for *chi*-driven GUS activity in different tissues and various cell types. In a similar procedure using chimeric *chi*A-GUS genes, the *chi*A tandem promoter regulation was investigated.

RESULTS

Construction of Chalcone Flavanone Isomerase (*chi*)-Reporter Gene Fusions

To investigate the tandem promoter regulation of *chi*A gene expression and to determine the organ and cell specificity of the various *chi* promoters (*chi*A promoters P_{A1} and P_{A2} and the *chi*B promoter P_B; see also Figure 1A), chimeric genes were constructed consisting of a *chi* promoter fused in front of the GUS reporter gene (Jefferson et al., 1987). These constructs were then introduced into petunia by *Agrobacterium tumefaciens*-mediated transformation. Transgenic plants were analyzed for *chi*-driven GUS expression in a fluorimetric assay or by histochemical analysis.

Four different GUS constructs were made: three fusions of GUS with various *chi*A promoter fragments (Figure 1B, constructs 1, 2, and 3) and one fusion with a *chi*B promoter fragment (Figure 1B, construct 4).

A 4.4-kb *chi*A 5′ fragment, spanning the XbaI site at position +195 (for sequence of *chi* genes, see van Tunen et al., 1989) to the 5′ XbaI site at −4200 and containing both the P_{A1} and P_{A2} transcription starts (at +1/+4 and −437, respectively), was fused in the correct reading frame to the GUS reporter gene (Figure 1B, construct 1). This chimeric gene contains 56 CHI triplets fused in front of the GUS coding region. Removal of the sequence upstream of the proximal HindIII site (at position −437) yielded construct 2 containing a 0.6-kb P_{A1} promoter fragment (Figure 1B). A 0.44-kb HindIII fragment from the *chi*A P_{A2} promoter (Figure 1A) was isolated and engineered in front of the GUS coding region, rendering a transcriptional fusion product (Figure 1B, construct 3). A 1750-bp *chi*B promoter fragment (ranging from an EcoRI site at −1700 to position +62) was fused in frame with the GUS coding sequence, yielding a translational fusion product containing 11 *chi*B amino acids (Figure 1B, construct 4).

Tandem Promoter Regulation of the *chi*A Gene; Temporal and Spatial Activity of the *chi*A P_{A1} Promoter

To investigate whether sequences upstream of the P_{A2} transcription start (Figure 1A) are involved in determining the proper temporal and spatial specificity of the *chi*A P_{A1} promoter, the GUS activity in different tissues of P_{A1}/P_{A2}-GUS or P_{A1}-GUS transformants (Figure 1B) was determined. Fluorimetric analysis showed that 10 out of 15 independent *chi*A P_{A1}/P_{A2}-GUS and two out of six independent *chi*A P_{A1}-GUS-transformed plants expressed *chi*A-specific GUS activity in flower limbs, flower tubes, and leaves, but not in mature anthers (data not shown).

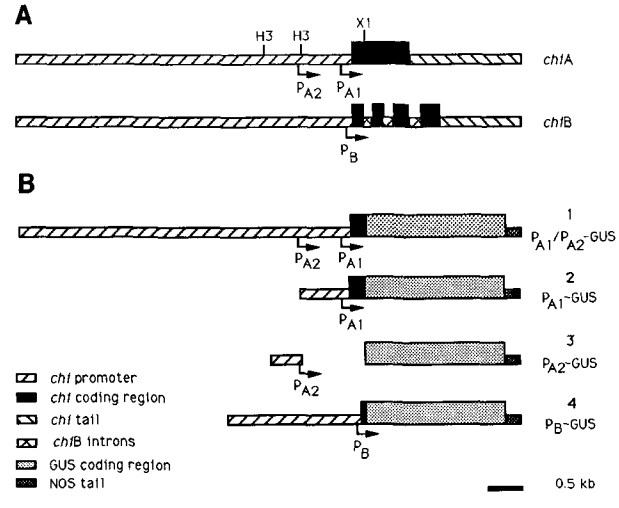

Figure 1. Construction of *chi*-GUS Chimeric Genes.

(A) Structure of petunia V30 CHI genes. The structural parts of the genes are shown as solid black bars; bent arrows represent the transcription starts. Restriction enzyme sites used for fusion with the GUS coding region are shown: H3 = HindIII; X1 = XbaI.
(B) *chi*-GUS chimeric gene constructs. *chi*-GUS chimeric genes consisted of a *chi*A or *chi*B promoter region, part of the *chi*A or *chi*B gene coding region (translational fusions; constructs 1, 2, and 4) or no coding part (transcriptional fusion; construct 3), the GUS coding region and the NOS tail. Bent arrows represent the various transcription starts.

Table 1. Use of *chi*A P_{A1}/P_{A2}(P_{A1}/P_{A2}-GUS in Transformant VRD3) and *chi*A P_{A1} (P_{A1}-GUS in Transformant VRE3) Promoter in Different Tissues of Transformed or Untransformed (VR Control) Petunia Plants

Tissue[a]	Specific GUS Activity		
	P_{A1}/P_{A2}-GUS	P_{A1}-GUS	VR Control
	pmol 4-Mu/min/mg protein[b]		
Limb	601	366	35
Tube	490	62	24
Anther	66	77	61
Pistil	211	156	210
Seed[c]	566	1603	28
Sepal	1078	103	49
Leaf	2311	533	11
Stem	201	172	35

[a] All flower tissues are taken from buds of developmental stage 4 (fully mature but before anthesis).
[b] 4-Mu, 4-methylumbelliferone.
[c] Ten days after (self) pollination.

Although the remaining five P_{A1}/P_{A2}-GUS and four P_{A1}-GUS plants were transformed, no GUS activity above the background was measured in any of their tissues tested (flower limb and tube, leaf, and anther).

One transformant for each construct (VRD3 and VRE3) was selected and analyzed in detail for GUS activity in various tissues. The results are shown in Table 1. Both the P_{A1}/P_{A2}-GUS and the P_{A1}-GUS constructs gave rise to *chi*A-driven GUS activity in flower limb, flower tube, seed, sepal, leaf, and stem. Neither P_{A1}/P_{A2}- nor P_{A1}-driven GUS expression was observed in mature anthers or pistils of transgenic plants. Similar results were obtained for transgenic W115 petunia plants containing the same constructs (data not shown). Although the P_{A2} promoter is activated late in anther development, this promoter activity in a P_{A1}/P_{A2}-GUS construct is not reflected in GUS enzyme activity because the first ATG 3′ of the P_{A2} transcription start (at position −257) is followed by a TAG stop at position −227, which gives rise to a non-sense protein.

The cell specificity of the P_{A1} promoter was determined by histological techniques. No differences in cell specificity between the P_{A1}/P_{A2} and the P_{A2} promoter fragments were observed when P_{A1}/P_{A2}-GUS and P_{A1}-GUS transgenic plants were analyzed histochemically for *chi*A-driven GUS activity. In Figures 2B to 2F histochemical analyses of a *chi*A P_{A1}/P_{A2}-GUS transformed plant (VRD3) is shown. In flower limbs the P_{A1} promoter is active in both epidermal cell layers (Figure 2B, IE and OE) and also in the parenchyma cells in between, whereas no activity is observed in limbs of untransformed petunia plants (Figure 2A). In tubes of flower buds, P_{A1}-driven GUS expression is detected in the inner epidermal cells (IE), the trichomes, and in parenchyma cells immediately underneath the epidermal cell layer (Figure 2C). Ten days after pollination, a high level of P_{A1}-driven GUS activity was detected in a seedpod, whereas a seedpod of an untransformed control plant is unstained (Figure 2D). Figures 2E and 2F show that P_{A1}-driven GUS activity is found in the embryo cells, endosperm, and seed coat. Only low levels of GUS activity were detected in placenta cells.

Pollen-Specific *chi*A P_{A2} Promoter Activity

Activation of the P_{A2} *chi*A promoter occurs late in anther development and leads to the accumulation of a 1.5-kb *chi*A transcript in mature anthers and more specifically in pollen grains. To investigate whether a 0.44-kb HindIII *chi*A fragment contains sufficient regulatory sequences to direct pollen-specific gene expression, construct 3 (Figure 1B) was introduced into petunia W115 and the VR hybrid. Ten independent transgenic plants were raised and analyzed fluorimetrically for P_{A2}-driven GUS activity. Four transformants showed GUS activity in mature anthers and pollen grains, whereas the remaining six transgenic plants did not express GUS activity above background in any tissue tested (flower limb and tube, leaf, stem, and anthers).

One P_{A2}-GUS transformant (VRF1) was selected and analyzed fluorimetrically in detail for GUS enzyme activity in various tissues. Table 2 shows that P_{A2}-driven GUS activity was only detectable in mature anthers and pollen grains. No GUS activity was detected in flower limb, flower tube, ovarium, leaf, or stem. In Figure 3, fluorimetric analysis of another transformant (WF5) confirmed these data and showed that during the maturation of the anthers the P_{A2} promoter drove the GUS reporter gene in a developmental way parallel to the accumulation of the 1.5-kb *chi*A P_{A2} transcript.

Histochemical analysis of a P_{A2}-GUS transgenic plant confirmed the presence of GUS activity in pollen grains (Figure 2H). Although endogenous GUS activity is present in mature anthers, and especially in the pollen grains (Plegt and Bino, 1989), under our staining conditions only low levels of GUS activity were observed in pollen grains from an untransformed control (Figure 2G). Analysis of in vitro germinated pollen showed the presence of GUS activity in the cytoplasm of pollen tubes from the P_{A2}-GUS transformant (Figure 2J), whereas only a low degree of blue stain was observed in pollen tubes from a control (Figure 2I).

Analysis of another P_{A2}-GUS transformant (WF5; see also Figure 3) confirmed the histochemical data. After self-pollination of this P_{A2}-GUS transformant or after pollination of this transformant with pollen from an untransformed control, no GUS activity could be detected fluorimetrically in the seeds 7 days after pollination (data not shown).

Taken together these data show that P_{A2} is a pollen-specific promoter activated late in anther development.

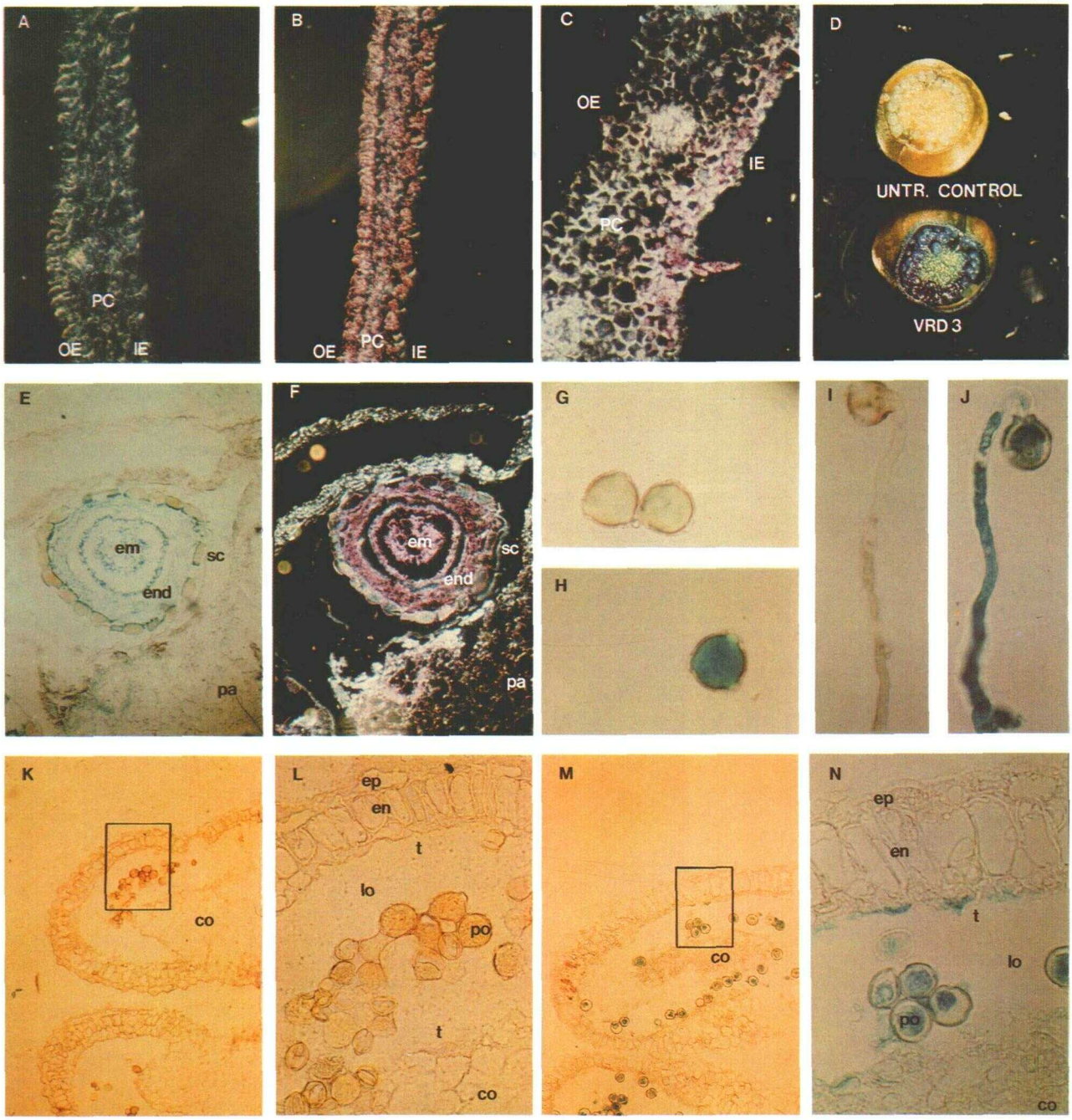

Figure 2. Histochemical Analysis of *chi*-Driven GUS Expression.

The abbreviations used are: co, connective; en, endothecium; end, endosperm; em, embryo; ep, epidermis; IE, inner epidermis; lo, lobule; OE, outer epidermis; pa, placenta; PC, parenchyma cells; po, pollen grains; sc, seed coat; t, tapetum.

(A) Cross-section of a limb (developmental stage 3) of an untransformed VR plant; dark-field picture.

(B) Cross-section of a limb (developmental stage 3) of VRD3 transgenic plant containing P_{A1}/P_{A2}-GUS construct; dark-field picture.

(C) Cross-section of tube (developmental stage 3) of VRD3 transgenic plant containing P_{A1}/P_{A2}-GUS construct; dark-field picture.

(D) Macroscopic picture of seedpod (10 days after pollination) of untransformed VR plant (top) or from VRD3 transgenic plant containing the construct P_{A1}/P_{A2}-GUS (bottom).

(E) and **(F)** Microscopic picture of a seed from a VRD3 transgenic plant containing the P_{A1}/P_{A2}-GUS construct. **(E)** bright-light picture, **(F)** dark-field picture.

Table 2. *chi*A Promoter P_{A2} Activity in Different Tissues of Transformed (P_{A2}-GUS in Transformant VRF1) or Untransformed (VR Control) Petunia Plants

Tissue[a]	Specific GUS Activity	
	P_{A2}-GUS	VR Control
	pmol 4-Mu/min/mg protein	
Limb	98	35
Tube	54	24
Anther	1424	61
Pollen	670	27
Ovarium	94	128
Leaf	36	11
Stem	46	35

[a] All flowers are from buds of developmental stage 4 (fully mature but before anthesis) except for anthers and pollen, which were from open flowers with anthers at anthesis.

Anther-Specific *chi*B P_B Promoter Activity

*chi*B gene promoter (P_B) activity results in the accumulation of a 1.0-kb RNA transcript in immature anthers of developmental stages 2 and 3. To investigate the temporal and spatial specificity of the P_B promoter, construct 4 (Figure 1B) was introduced into both petunia (VR hybrid) and tobacco (SR1). Seven independent transgenic VR plants and seven independent SR1 plants were raised and analyzed fluorimetrically for P_B-driven GUS activity. One petunia and two tobacco transformants showed *chi*B-driven GUS activity in immature anthers. This petunia transformant (VRB8) and one tobacco P_B-GUS transformant (TAB-2) were analyzed in more detail. Table 3 indicates that both showed GUS enzyme activity only in immature anthers but not in limb, tube, pistal, ovarium, seed, sepal, leaf, or stem.

Figure 3 shows that the amount of P_B-driven GUS activity parallels the accumulation of *chi*B mRNA during anther development and peaks in anthers of developmental stage 2/3 in both petunia and tobacco.

The cell specificity of the P_B promoter in anthers (developmental stage 3) from P_B-GUS transgenic petunia plants was determined in a histological assay. P_B-driven GUS activity was detected in the pollen grains and tapetum cells of transgenic anthers (Figures 2M and 2N), whereas no GUS activity was detected in those cells of an untransformed control plant (Figures 2K and 2L).

DISCUSSION

We have analyzed the spatial and temporal activities of chalcone flavanone isomerase (*chi*) promoters of petunia. The tandem promoter regulation of the *chi*A coding region and the use of *chi*A and *chi*B promoters in the regulation of anther- and pollen-specific gene expression were investigated. We determined the cell specificity of the various *chi* promoters.

*chi*A Tandem Promoter Regulation

In a previous paper we reported the accumulation of two different *chi*A transcripts in floral tissues of petunia; a 1.0-kb transcript accumulates in petals, whereas a 1.5-kb *chi*A transcript accumulates in pollen grains (van Tunen et al., 1988). A combination of RNase protection experiments and primer extension analyses strongly suggests that the *chi*A coding region is regulated by a double promoter. The use of promoter P_{A1} leads to the accumulation of the 1.0-kb *chi*A transcript, whereas the upstream P_{A2} promoter results in the synthesis of the 1.5-kb *chi*A transcript. In this paper we describe experiments in which we have separated the P_{A1} and P_{A2} transcription starts and fused the resulting *chi*A promoter fragments to GUS. Each promoter fragment is capable of driving the GUS reporter gene (Tables 1 and 2, Figure 2), which provides definitive proof for the existence of two distinct *chi*A promoters. Furthermore, we show that both a 0.6-kb P_{A1} and a 0.44-kb P_{A2} promoter fragment drive GUS activity in parallel with the accumulation of the 1.0-kb P_{A1} and 1.5-kb P_{A2} transcripts, respectively (Tables 1 and 2, Figure 3). We conclude that sequences upstream of the P_{A2} transcription start are not necessary for the proper spatial and temporal activities of the P_{A1} promoter. Similarly, the P_{A2} promoter functions independently of the P_{A1} promoter sequences. For a number of animal and prokaryotic genes, tandem promoter regulation of gene expression has been reported before (for review, see Schibler and Sierra, 1987). In plants, tandem promoter regulation has been suggested for a

Figure 2. (continued).

(G) and **(H)** Microscopic picture of mature pollen grains from flowers (developmental stage 6) of untransformed VR plant **(G)** or transgenic VRF1 plant **(H)** containing the P_{A2}-GUS construct.

(I) and **(J)** Microscopic picture of in vitro germinated pollen grains from flowers (developmental stage 6) of untransformed VR plant **(I)** or transgenic VRF1 plant **(J)** containing the P_{A2}-GUS construct.

(K) and **(L)** Microscopic picture of anther of developmental stage 3 of an untransformed VR plant. **(K)** an overview, **(L)** a magnification of the boxed region of **(K)**.

(M) and **(N)** Microscopic picture of anther of developmental stage 3 of a transgenic VRB8 plant containing the P_B-GUS construct. **(M)** an overview, **(N)** a magnification of the boxed region of **(M)**.

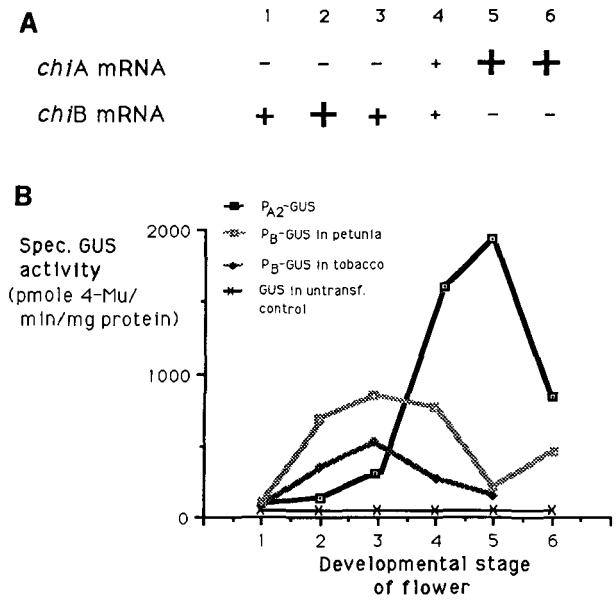

Figure 3. *chi* P_{A2}- and *chi* P_B-Driven GUS Activity in Anthers during Flower Development.

Anthers analyzed for *chi* RNA accumulation or expression of GUS activity were from flower buds or flowers of different developmental stages. Fully mature flower buds were defined as 100% flower buds. Stage 1, flower buds 25% of mature flower buds; stage 2, flower buds 25% to 50%; stage 3, flower buds 50% to 75%; stage 4, flower buds 75% to 100%; stage 5, open flowers/anthers before anthesis; stage 6, open flowers/anthers after anthesis (see also, van Tunen et al., 1988, 1989).

(A) Accumulation of *chi*A (1.5-kb P_{A2} transcript) or *chi*B RNA (1.0-kb P_B transcript) in anthers of different developmental stages. The amount of *chi* RNA corresponds to the size of the "+." Absence of *chi* RNA is indicated with a "−." Data represent an impression of published results (van Tunen et al., 1988, 1989).

(B) Fluorimetric analyses for GUS activity in transgenic P_B-GUS petunia (VRB8)/tobacco (TAB-2) plants and transgenic P_{A2}-GUS petunia plants (WF5). GUS activity in anthers of untransformed VR or W115 petunia or SR1 tobacco plants is summarized as a single line and does not exceed 37, 82, or 63 picomoles of 4-methylumbelliferone (4-Mu) per minute per milligram of protein, respectively.

number of genes (extensin genes: Chen and Varner, 1985; *A1*: Schwarz-Sommer et al., 1987; *Bz-1*: Furtek et al., 1988), but only convincingly shown in the case of a zein gene of maize (Brown and Feix, 1990; Quattrocchio et al., 1990) and *chi*A from petunia (this article). In contrast to the *chi*A promoters, the zein promoters are not differentially used but show the same temporal and spatial specificity.

A close coordination of the P_{A1} promoter, both in a temporally and spatially regulated way, with the expression pattern of the *chs*A and *chs*J promoters has been observed (Koes et al., 1990). Coordinate expression of the *chi*A P_{A1} promoter with *chs*A- and *chs*J-driven GUS activity was observed in limbs (epidermal and parenchyma cells), tubes (inner epidermal and parenchyma cells), and seedpods (seed coat cells, endosperm, and embryo). No *chi*A P_{A1}-driven GUS activity was detected in anthers of transformed VR plants. This is according to the *Po* recessive status of V30, the petunia line from which the *chi* genes were isolated (van Tunen, 1990). Because parenchyma cells of limbs are normally unpigmented, the activity of the *chi*A P_{A1} promoter in these cells was unexpected. Apparently, in these cell types flavonoids serve functions other than pigmentation (Koes et al., 1990). *chi*A P_{A1}-driven GUS activity was seen in leaves and stems of all transgenic plants expressing this activity in flowers (Table 1). This reflects the in vivo expression pattern of *chi* genes because low amounts of CHI enzyme activity have been detected in leaves of the same transgenic plants (data not shown). One should bear in mind that GUS is a relatively stable enzyme (Jefferson et al., 1987). Therefore, a low but constant promoter activity can result in significant amounts of GUS enzyme activity. In contrast, no *chs*-driven GUS activity was measured in leaves of transgenic petunia plants (Koes et al., 1990). We explain this difference in *chi*A P_{A1} and *chs* promoter activity by assuming that positive regulatory elements involved in the regulation of *chs* genes in leaf and stem might be absent in the *chs* promoter parts analyzed in these experiments. Alternatively, negative regulating elements preventing *chi*A P_{A1} promoter activity in leaf and stem can be absent in the *chi*A-GUS chimeric genes introduced into plants.

Table 3. *chi* Gene B P_B Promoter Activity in Different Tissues of Transformed Petunia (P_B-GUS in Transformant VRB8) and Tobacco (P_B-GUS in Transformant TAB-2) or Untransformed Petunia (VR Control) or Tobacco (SR1 Control) Plants

	Specific GUS Activity			
	Petunia		Tobacco	
Tissue[a]	P_B GUS	VR Control	P_B GUS	SR1 Control
	pmol 4-Mu/min/mg protein			
Limb	42	35	18	12
Tube	51	24	19	34
Anther	730	61	475	29
Pistil	161	210	81	61
Ovarium	NT[b]	128	11	16
Seed[c]	NT	28	41	37
Sepal	61	49	20	24
Leaf	NT	11	30	8
Stem	NT	35	26	22

[a] All flower tissues are from buds of developmental stage 4 (fully mature but before anthesis).

[b] NT, not tested.

[c] Fourteen days after (self) pollination.

The *chi*A P_{A2} Promoter Is Pollen Specific

Table 1 shows that the *chi*A P_{A2} promoter is exclusively used in mature pollen grains. Histochemical analysis confirmed the fluorimetric data; GUS activity was detected in pollen grains by staining with X-gluc. In developing male gametophytes, the bulk of the protein is synthesized after pollen germination and during tube growth utilizing the large quantities of pre-existing ribosomes, tRNAs, and mRNAs (Mascarenhas, 1989). However, the extraction of enzymatically active GUS protein from mature pollen grains containing P_{A2}-GUS implies that protein synthesis occurs in mature pollen grains. As in the mature male gametophyte, P_{A2}-driven GUS activity was also detected in the tube of pollen grains (Figure 2J). Whether this is the result of P_{A2} activity during the germination process or a reflection of the stability of the GUS enzyme is unclear. P_{A2} promoter activity is restricted to the mature male gametophyte and is absent in the developing seed as determined in pollination experiments using pollen from a P_{A2}-GUS expressor. The 1.5-kb *chi*A transcript resulting from the use of the *chi*A P_{A2} promoter contains an open reading frame with a 56-amino acid extension at the 5′ side. However, no larger CHI proteins could be detected after electrophoresis on SDS-PAGE of proteins synthesized after in vitro translation of RNA from mature anthers. Furthermore, no CHI enzyme activity could be demonstrated in the same tissue or in pollen grains. Finally, it was shown that the 1.5-kb *chi*A transcript is neither coordinately regulated with *chs* mRNA nor UV induced (these data have been published before; van Tunen et al., 1988, 1989). Therefore, the in vivo function, if any, of the *chi*A P_{A2} promoter is still obscure.

The *chi*B P_B Promoter Is Anther Specific

The *chi*B P_B promoter is activated early in anther development (anthers of developmental stage 2 and 3; see Figure 3) and more specifically in the microsporangia and tapetal cells (Figure 2). At these developmental stages, anthers have reached their maximum size, and the inner and outer tapetum starts to degenerate (Nave and Shawhney, 1986). P_B-driven GUS activity has been observed in none of the other tissues tested (Table 3). Taken together these data indicate that the P_B promoter is only active in anthers.

The spatial and temporal use of the P_B promoter resembles that of the *LAT52* tomato promoter that was described recently (Twell et al., 1989). Comparison of the 5′-controlling regions of both genes revealed a 9-bp perfect homology (CCACAAAAA) that was also present in the P_{A2} promoter region of *chi*A (van Tunen et al., 1989). However, the significance of the homology identified remains to be established by functional analysis (e.g., site-directed mutation experiments).

chi A P_{A2} and P_B promoters are specifically activated in distinct and different stages of male gametophyte development, suggesting the use of these promoters in the study and manipulation of the development of anthers and/or pollen. Experiments are now in progress in which chimeric genes are constructed that consist of fusions between the *chi*A P_{A2} and *chi*B P_B promoters with genes encoding products that interfere with primary metabolism or lead to the synthesis of plant hormones. Ultimately, this might provide a way to introduce nuclear-encoded male sterility in plants.

METHODS

Construction of *chi*-GUS Fusion Genes

Four chimeric genes consisting of a CHI promoter region and the GUS reporter gene with the NOS tail (Jefferson et al., 1987) were made. Either translational (using one of the GUS plasmids pBI101.1, -101.2, or -101.3, which differ from each other by 1 bp rendering different reading frames) or transcriptional fusions were made. DNA manipulations were carried out according to standard methods (Maniatis et al., 1982). All constructs were checked by restriction analysis. The fusion sites were checked by double-strand DNA sequencing using the GUS sequencing primer described by Jefferson et al. (1987). After mobilization of the constructs to the *Agrobacterium tumefaciens* strain LBA 4404 (Bevan, 1984), the cell culture used for transformation was checked again by restriction analysis for the occurrence of recombination events.

Plant Transformation

Because the *Petunia hybrida* line V30, from which the *chi* genes were isolated, is recalcitrant to transformation, the chimeric *chi*-GUS genes were introduced into other petunia varieties. As was shown earlier (van Tunen and Mol, 1987), CHI expression in petunia is regulated by the gene *Po*. The VR hybrid was used for transformation because the genotype of this variety is *Po* dominant and because this hybrid is well transformable (Wallroth et al., 1986).

P. hybrida VR (genotype *Po/po An2/An2*) or W115 plants (genotype: *po/po an2/an2*) or tobacco SR1 plants used for transformation were grown under standard greenhouse conditions. Transformants were obtained by the standard leaf disc transformation method (Horsch et al., 1985). Leafs discs were prepared from top leaves of young, nonflowering plants. After cultivation with *A. tumefaciens* (LBA 4404), petunia leaf discs were grown on Murashige and Skoog (MS) (1962) plates (Horsch et al., 1985) containing 2 μg/mL benzylaminopurine, 0.01 μg/mL naphthaleneacetic acid, 500 μg/mL carbenicillin, and 250 μg/mL kanamycin, whereas tobacco leaf discs were grown on MS plates containing 3 μg/mL kinetin, 500 μg/mL carbenicillin, and 200 μg/mL kanamycin. All plants raised were checked for transformation on the basis of resistance for kanamycin. (Leaf discs were taken from plants and grown on MS plates supplemented with hormones in the presence or absence of kanamycin.) DNA gel blot analysis showed the presence of 1 to 3 copies, on average, of each construct per genome.

GUS Extractions and Fluorimetric and Histochemical GUS Assays

Fresh material was collected from the transgenic plants and used for GUS extractions. GUS extractions were performed as described by Jefferson et al. (1987) by grinding the tissue with sand and Dowex-1 (Sigma) or with liquid N_2 and Dowex-1. For extraction of GUS activity from pollen grains, grinding in liquid N_2 was essential.

GUS activity was quantified by fluorimetric analysis and calculated according to Jefferson et al. (1987).

For histochemical analysis fresh tissues were cut into slices with a razor blade and stained with 1 mM X-gluc (Research Organics Inc.) at 37°C for 3 hr to 10 hr in 50 mM Na-phosphate, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide. After fixation in 50 mM Na-phosphate, pH 7.0, 1% glutaraldehyde, 1% formaldehyde for 16 hr, the tissues were dehydrated by a passage through a series of ethanol solutions (70%, 4 hr to 16 hr; 80%, 30 min; 90%, 30 min; 96%, 30 min; and 100%, 30 min), amyl acetate/ethanol 1:1 (30 min), amyl acetate (30 min), and liquid parafin (2 × 30 min at 50°C). After embedding in the paraffin 7-μm-thick slices were cut using a microtome. Slices were put on a microscope slide with a drop of 1% glycerin (Merck), dried for 16 hr at 37°C, treated for 5 min with xylene to remove paraffin, mounted with malinol (Merck) and a cover, and allowed to harden overnight. Photographs were taken by light-field or dark-field microscopy.

To exclude artifacts, which can result from differences in cell size, penetration of substrate into the tissue, and background enzyme activity, we repeatedly performed the histochemical assays on floral organs of transgenic, untransformed, and control plants (the latter containing the cauliflower mosaic virus 35S/GUS gene construct made by Jefferson et al., 1987). Pollen grains and germinated pollen were stained for 3 hr with X-gluc and analyzed without making a cross-section. Germination of the pollen was performed according to Bino et al. (1987).

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