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Antisense Inhibition of Flavonoid Biosynthesis in Petunia Anthers Results in Male Sterility

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Inhibition of flower pigmentation in transgenic petunia plants was previously accomplished by expressing an antisense chalcone synthase (*chs*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This chimeric gene was not effective in inhibiting pigmentation in anthers, presumably because the viral CaMV 35S promoter was insufficiently expressed in cell types of this organ in which the pigments are produced. Insertion of the anther box, a homologous sequence found in other genes expressed in anthers, resulted in a modified expression pattern driven by this promoter, as monitored by the β-glucuronidase (*gus*) gene. In addition to the basic CaMV 35S expression pattern in anthers, GUS activity was observed in tapetum cells when the modified promoter was fused to the *gus* gene. This promoter construct was subsequently used to drive an antisense *chs* gene in transgenic petunia, which led to the inhibition of pigment synthesis in anthers of five of 35 transformants. Transgenic plants with white anthers were male sterile due to an arrest in male gametophyte development. This finding indicated that flavonoids play an essential role in male gametophyte development.

INTRODUCTION

In most flowers, the pigments are composed of flavonoids. Because pigmentation is such a convenient phenotypic marker, the biochemistry and genetics of flavonoid biosynthesis have been studied extensively (Heller and Forkmann, 1988; Forkmann, 1991). In the last several years, a large number of the genes involved have been cloned and characterized (for reviews, see Forkmann, 1991; van Tunen and Mol, 1991).

Flavonoids are phenylpropanoid-based secondary metabolites that, besides their role in the pigmentation of flowers and fruit, display many other functions. They are involved in the defense against phytopathogens in plant species such as French bean and soybean (Dixon, 1986; Lamb et al., 1989), the protection against UV light (Schmelzer et al., 1988), and the induction of nodulation in legumes (Long, 1989). They also have been implicated in the regulation of auxin transport (Jacobs and Rubery, 1988) and resistance to insects (Hedin and Waage, 1986).

Chalcone synthase (CHS) is the key enzyme of flavonoid biosynthesis. The temporal and spatial regulation of *chs* genes in petunia was recently studied by way of two different approaches: by the introduction of chimeric genes consisting of *chs* promoters fused to the β-glucuronidase (*gus*) reporter gene (Koes et al., 1990) and by the introduction of antisense *chs* genes (van der Krol et al., 1988, 1990a, 1990b). The expression of an antisense *chs* cDNA driven by either the constitutive cauliflower mosaic virus (CaMV) 35S promoter (van der Krol et al., 1988) or by the homologous *chs* promoter (van der Krol et al., 1990b) leads to a dramatic decrease in corolla pigmentation in 10 to 50% of the transformants. However, the pigmentation of the anthers is unaffected. Analysis of the expression of promoter-*gus* fusions showed that the *chs* promoter drives expression only in the tapetum cell layer and in the outer parenchymatic cells of the connectivum (Koes et al., 1990). The CaMV 35S promoter on the other hand shows expression in almost every cell type of the anther except for the tapetum and sporogenous tissues (Plegt and Bino, 1989). The principal site of flavonoid synthesis in the anther is the tapetum (Herdt et al., 1978; Kehrel and Wiermann, 1985; Beerhues et al., 1989), from which the pigments are transported into the locule and incorporated in the outer surface of the pollen grain (Wiermann and Vieth, 1983). At later stages of anther development, flavonoids are distributed throughout the anther, and it is thought, therefore, that transport of flavonoids (or their precursors) may occur not only into the locule but also further into the anther (for transport of flavonoids, see Hrazdina and Wagner, 1985; Knogge and Weissenböck, 1986; Koes et al., 1990). Consequently, the failure to block anther pigmentation by way of the CaMV 35S or *chs*-driven antisense *chs* gene expression could

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reside in the incorrect expression pattern and/or an insufficient expression level.

To study the possible role of flavonoid compounds in anther development, we attempted to down regulate flavonoid synthesis in the tapetum by changing the cell-type specificity of the CaMV 35S promoter–driven antisense *chs* gene expression. For this purpose, we modified the CaMV 35S promoter by inserting one, two, or eight copies of the anther box, a homologous sequence present in flavonoid-specific genes active in immature anther tissue (van Tunen et al., 1988).

Reporter gene expression driven by this modified CaMV 35S promoter in transgenic petunia demonstrated that it acquires the desired tapetum specificity on top of the basic expression pattern. We showed further that this chimeric promoter fused to antisense *chs* cDNA is able to inhibit flavonoid synthesis in anther tissue and pollen of transgenic petunia plants. The absence of flavonoids in the male gametophyte had a profound effect on its development: pollen maturation was arrested, resulting in male sterile plants. These results indicated that flavonoid biosynthesis is essential to normal pollen development.

RESULTS

Effect of the Anther Box on Gene Expression Driven by the CaMV 35S Promoter in Anthers

In previous experiments in which the CaMV 35S promoter was used to drive antisense *chs* cDNA expression, inhibition of pigmentation in anthers was never observed. These results might be explained by assuming that the expression driven by the CaMV 35S promoter is either relatively low or exhibits a different temporal and/or spatial regulation compared with the endogenous *chs* gene expression in anthers. Plegt and Bino (1989) showed that the CaMV 35S–driven expression pattern of the *gus* gene in anthers is diffuse and that the tapetum cells exhibit no activity. Because the tapetum cells are the principal site of flavonoid biosynthesis in anthers (Kehrel and Wiermann, 1985; Beerhues et al., 1989), we aimed at modification of the cell-type specific expression of the strong CaMV 35S promoter to achieve antisense inhibition of anther pigmentation.

Figure 1 shows a comparison of the promoters of flavonoid genes that are active in immature anther tissue of the petunia cultivar V30 (*chs*A, *chs*J: Koes et al., 1989; chalcone flavanone isomerase [*chi*] B gene: van Tunen et al., 1989; dihydroflavonol-4-reductase [*dfr*] A gene: Beld et al., 1989). The presence of a strongly conserved sequence is evident (anther box: van Tunen et al., 1989, 1990). The CaMV 35S promoter was modified by inserting one, two, or eight copies of a synthetic double-stranded oligonucleotide corresponding to the anther box sequence present in the *chs*A promoter (position −238 to −221) at position −90 into the EcoRV site.

To be able to analyze the influence of the anther box on the spatial expression driven by the CaMV 35S promoter, the chimeric CaMV 35S/anther box promoter (containing eight copies of the anther box sequence in direct repeats) was cloned in front of the *gus* reporter gene. This chimeric promoter and the normal CaMV 35S promoter of VIP102 (van der Krol et al., 1988) fused to the *gus* reporter gene were terminated by the *chs* 3′ untranslated region. Figure 2 shows the construction of this chimeric gene.

		Sst I	PstI
Synthetic Anther Box		GAGCTCTAGAGGTGACAGAAATCTGCAG	
chs A	(-250)	tcaaagcTGaTgcTAGAGGTGACAgAAATCatatgca	
chi B	(-640)	GATGCAATGTTATTAGAGGTGACAAAAATCGACCCAA	
chs J	(-332)	GATGCAtgGTTAaT.GAGGaGAaAAAgATtGACCaAA	
dfr A	(-896)	tATGactTaTccTTAGAGGaGAagtAAtagacgaCAA	

Figure 1. Sequence of the Synthetic *chs*A Anther Box and Comparison of the Promoter Regions of Flavonoid Genes Active in Immature Anther Tissue.

From top to bottom: synthetic *chs*A anther box sequence used in this study; anther box sequence found in the promoters of *chs*A, *chi*B, *chs*J, and *dfr*A, respectively. Capital letters represent nucleotides homologous to the sequence of the *chi*B gene, which is only expressed in the tapetum cells of young anthers (van Tunen et al., 1990).

Twenty-four independent transgenic petunia plants containing the CaMV 35S/anther box–*gus* construct (pTS24) and 25 transformants containing the control CaMV 35S–*gus* construct (pTS23) were analyzed. Because the expression level of an introduced gene can differ between independent transformants due to the so-called position effect (for a review, see Weising et al., 1988), the *gus* expression in anthers was measured relative to that in corolla tissue for all the individual transformants. The analyses were performed in triplicate for each transformant. Figure 3 shows the average ratio of GUS activities in anthers and corollas for the individual plants that expressed the transgene. The ratios of GUS activity in anthers versus corolla were significantly higher in transformants containing the CaMV 35S/anther box–*gus* construct compared to those containing the control CaMV 35S construct, as was tested with the Wilcoxon's rank test with $\alpha < 0.05$. The presence of eight copies of the anther box enhances CaMV 35S–driven GUS activity in anthers approximately threefold relative to that in corolla (Figure 3).

Presence of the Anther Box Influences the Cell-Type Specificity of the CaMV 35S Promoter in Anthers

To determine whether the insertion of the anther box into the CaMV 35S promoter alters its spatial expression, GUS expression was monitored histochemically in cross-sections of anthers.

A petunia anther comprises, from inside to outside, a vascular cylinder surrounded by parenchymatic cells of the connectivum, four loculi containing the sporogenic tissue (pollen), and the endothecium. Each of the loculi is surrounded

by a layer of specific cells (tapetum) that functions in nourishment of sporogenic cells. In late stages of anther development, the loculi are disrupted at the stomium and the pollen grains are released (Drews and Goldberg, 1989).

Cross-sectioned anthers of all independent CaMV 35S–*gus* transformants were assayed two to five times for GUS activity. Figure 4A shows that the CaMV 35S promoter drove *gus* expression in the vascular cylinder, connectivum, and the endothecium. Examination of anthers transformed by CaMV 35S–*gus* at the single-cell level showed the absence of GUS activity in the tapetum cells, in accordance with Bino et al. (1989; Figure 4B). Staining was not observed in anthers of untransformed control plants (data not shown). Histochemical analysis of all independent transformants expressing the *gus* gene driven by the modified CaMV 35S promoter (pTS24) showed a slightly different GUS staining pattern in anthers (Figure 4C). In addition to the basic CaMV 35S–*gus* staining pattern, we observed blue staining of the tapetum cells. The tapetal localization of the GUS activity was more obvious at higher magnification (Figure 4D).

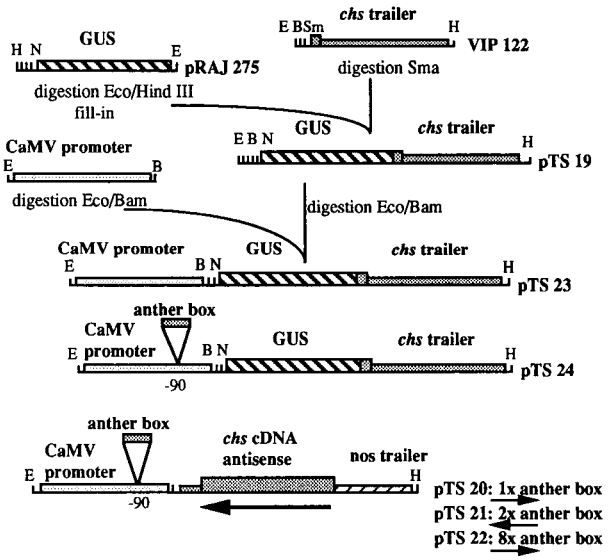

Figure 2. Construction of the Chimeric *gus* and Antisense *chs* Genes.

The chimeric *gus* constructs (pTS23 and pTS24) were generated by cloning the *gus* coding region of the pRAJ275 (Jefferson et al., 1987) as an EcoRI-HindIII fragment (filled in with the Klenow fragment) into the SmaI site of VIP122 that contains the *chsA* 3′ untranslated region (van der Meer et al., 1990), yielding pTS19. The CaMV 35S promoter of VIP102 (van der Krol et al., 1988) digested with EcoRI and BamHI was inserted into pTS19 cut with EcoRI and BamHI, resulting in pTS23. A synthetic anther box, homologous to the anther box present in the *chsA* promoter, was cloned (eight copies in the normal orientation) into pTS23 digested with EcoRV, yielding pTS24. The anther box was also cloned into the EcoRV site in the CaMV 35S promoter of VIP102 (CaMV–antisense *chsA nos*; van der Krol et al., 1988). The following antisense *chs* constructs were introduced in petunia plants: pTS20 (with a single anther box in the normal orientation), pTS21 (with two copies of the anther box in the reverse orientation), and pTS22 (with eight copies of the anther box in the normal orientation). Restriction enzyme sites are abbreviated as follows: B, BamHI; E, EcoRI; H, HindIII; N, NcoI; Sm, SmaI.

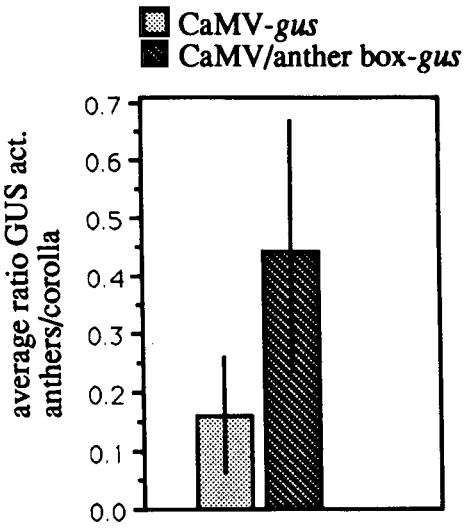

Figure 3. Analysis of *gus* Expression in Anthers and Corolla.

GUS activity was measured in anthers and corolla (stage 2, bud length: 15 to 25 mm) of all independent transgenic plants, and their ratios were averaged for the CaMV–*gus* transformants (25 plants; shaded bar) and CaMV/anther box–*gus* transformants (24 plants; hatched bar). The ratios differ significantly, as judged from the Wilcoxon's rank test ($\alpha < 0.05$).

Insertion of the Anther Box in the CaMV 35S Promoter Driving Antisense *chs* Expression Causes Inhibition of Anther Pigmentation

Assuming that high expression of the antisense *chs* transgene in the proper cell type (tapetum cells) of the anther would lead to reduction of pigmentation, the CaMV 35S promoter containing the anther box was used to drive antisense *chs* cDNA expression. To monitor possible effects of anther box copy number and/or orientation, the anther box was inserted at position −90 in the normal (one copy in pTS20 and eight copies in pTS22) and reverse (two copies in pTS21) orientations. Thirty-five independent petunia (VR hybrid) transformants were obtained, of which five plants yielded flowers with reduced anther pigmentation (Figure 4E). There was no correlation between inhibition of corolla pigmentation and that of the anthers. All four combinations of pigmented and unpigmented anthers and corollas were found (Figure 4F). Table 1 shows that transformation of the petunia VR hybrid with the pTS20 construct (one copy of the anther box in the normal orientation) resulted in 11 independent transgenic plants, of which five plants showed a normal phenotype, five plants showed reduced pigmentation of the corolla, and one plant yielded flowers with colored corolla and white anthers. Transformation with pTS21 (two copies of the anther box in reverse orientation) resulted in 15 independent transformants, of which nine plants showed no

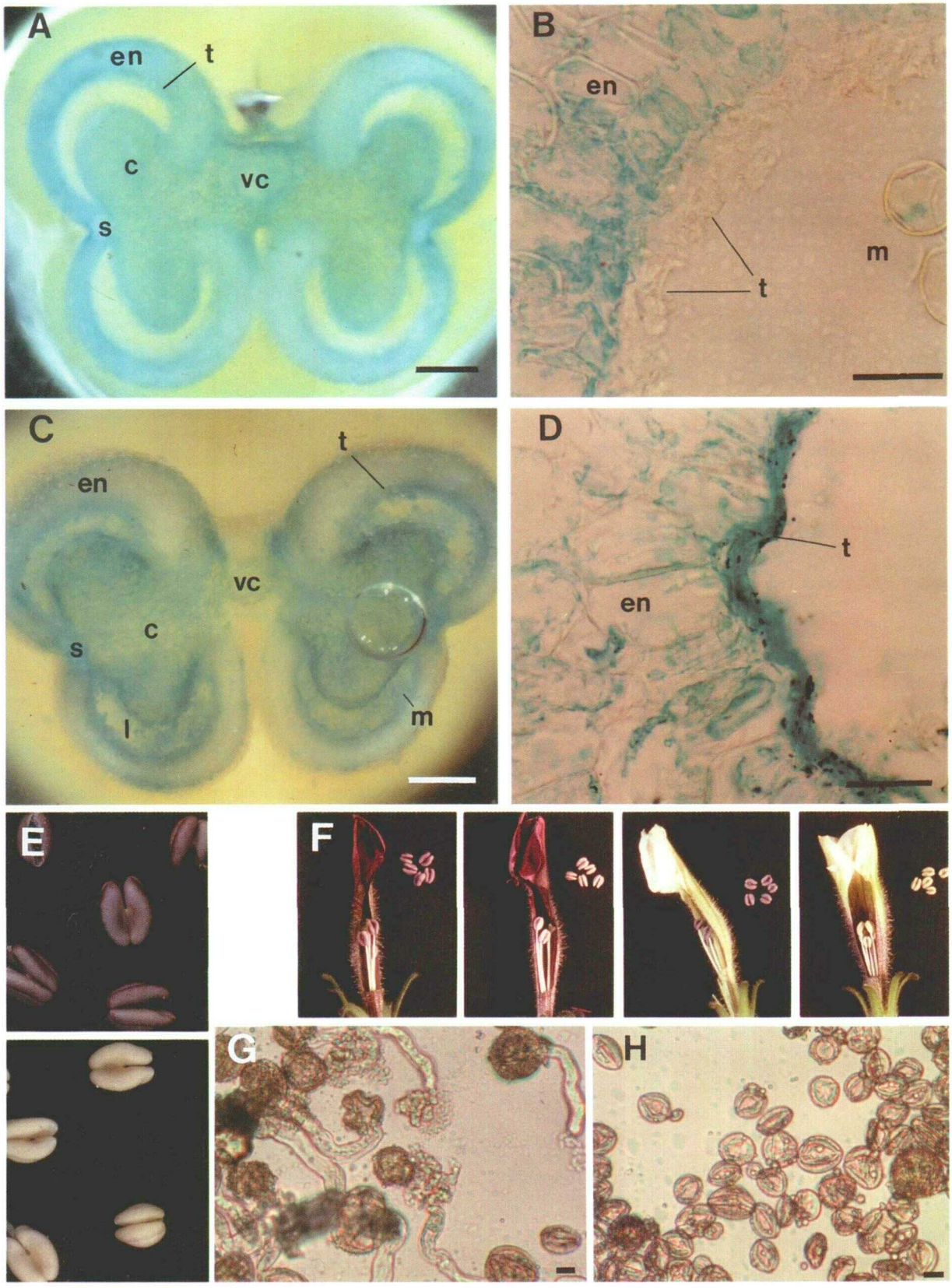

Figure 4. Effect of Modifying the CaMV 35S Promoter on GUS Activity in Anthers and on Antisense *chs* Inhibition in Anthers and Pollen.

Table 1. Distribution of the Antisense Phenotypes of Corolla and Anthers among the Different Transformants

	Phenotype				
Corolla/ Anthers	Purple/ Purple	White/ Purple	Purple/ White	White/ White	Total[a]
pTS20	5	5	1	0	11
pTS21	9	3	1	2	15
pTS22	7	1	1	0	9

[a] Total number of transformants.

effect, five plants gave rise to flowers with reduced corolla pigmentation of which two also had white anthers, and one transformant showed wild-type colored flowers with white anthers. Of the nine independent transformants containing pTS22 (with eight copies of the anther box in the normal orientation), seven plants had a wild-type phenotype, one plant showed flowers with reduced corolla pigmentation, and one plant bore flowers with white anthers and normally colored corollas.

Thin-layer chromatography of extracts from both white corollas and white anthers showed a severe reduction in flavonoid content accompanied by a twofold to threefold accumulation of phenylpropanoids such as coumaric acid and caffeic acid, which are precursors of flavonoid biosynthesis (data not shown).

Block in Anther Pigmentation Is Caused by a Reduction of the Steady-State *chs* mRNA Levels

It has been shown previously that antisense *chs* transgene expression may result in reduction of corolla pigmentation caused by a specific reduction of the *chs* mRNA steady-state level in the corolla. We examined whether the reduction of anther pigmentation resulted from a reduction of the *chs* mRNA level in anthers. To discriminate between the endogenous *chs* mRNA and the introduced antisense *chs* RNA, total RNA was isolated from white and purple anthers and analyzed by RNase protection. Because the *chs* DNA probe used originates from petunia cultivar V30 and *chs* genes from different petunia lines show some divergence, the endogenous VR *chs* transcripts are digested into four subfragments (van der Krol et al., 1990c). Figure 5 shows that inhibition of anther pigmentation is caused by a specific reduction (>90%) of the *chs* mRNA steady-state level in anthers. Plants that showed no phenotype had no reduction in *chs* mRNA levels in anthers (Figure 5, lane 2).

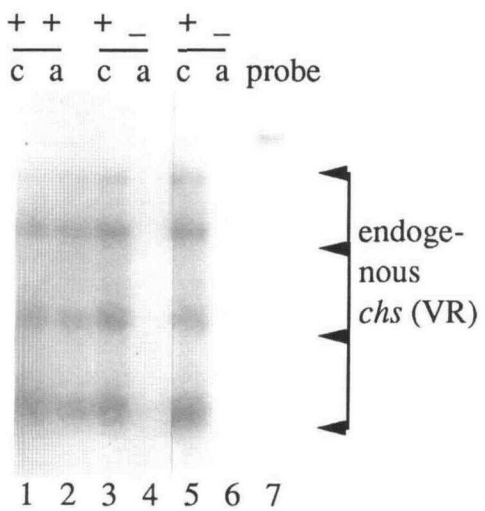

Figure 5. RNase Protection Analysis of Endogenous *chs* mRNA in Anthers and Corolla of pTS20 (CaMV/Anther Box–Antisense *chs* cDNA) Transformants.

RNA was extracted from corolla (c) and anthers (a) of transformants exhibiting a wild-type (+) corolla and a wild-type (+) anther pigmentation (pTS20.12, lanes 1 and 2) or a wild-type (+) corolla and a reduced (−) anther pigmentation (pTS20.4, lanes 3 and 4; pTS21.15, lanes 5 and 6). The petunia V30 *chs* cDNA antisense probe, used in the protection analysis, is shown in lane 7 (probe). Protection of the petunia V30 probe with petunia VR *chs* RNA results in four subfragments (see text).

Figure 4. (continued).

(A) Macroscopic image of a transverse section of an anther from a pTS23 (CaMV 35S–*gus*) transformant stained for 15 min with X-gluc. Bar = 300 μm.
(B) Magnification of a bright-field micrograph of a transverse section of an anther from a pTS23 (CaMV 35S–*gus*) transformant stained for 15 min with X-gluc. Bar = 30 μm.
(C) Macroscopic image of a transverse section of an anther from a pTS24 (CaMV 35S/anther box–*gus*) transformant stained for 15 min with X-gluc. Bar = 300 μm.
(D) Magnification of a bright-field micrograph of a transverse section of an anther from a pTS24 (CaMV 35S/anther box–*gus*) transformant stained for 15 min with X-gluc. Bar = 30 μm.
(E) Images of purple anthers (top) and white anthers (bottom) from a pTS21 (CaMV 35S/anther box–antisense *chs* cDNA) transformant.
(F) Four different phenotypes found among the CaMV 35S/anther box–antisense *chs* cDNA transformants. From left to right: colored corolla, colored anthers; colored corolla, white anthers; white corolla, colored anthers; white corolla, white anthers.
(G) In vitro germination of purple fertile pollen. Bar = 10 μm.
(H) In vitro germination of white sterile pollen of a pTS22 (CaMV 35S/anther box–antisense *chs* cDNA) transformant. Bar = 10 μm.
c, connectivum; en, endothecium; l, loculus; m, microspore; s, stomium; t, tapetum; vc, vascular cylinder.

Transformants with a Reduced Anther Pigmentation Are Male Sterile

Transformants with unpigmented anthers failed to produce mature seed pods after self-pollination. If some seeds were produced, they did not germinate (data not shown). To produce F_1 material of the antisense *chs* transformants, pollen of petunia (V30) was used in cross-pollination. About 50% of the progeny of the petunia V30 × VR hybrid cross should produce colored pollen. Plants in the F_1 progeny that inherited white pollen due to the presence of a *chs* transgene were examined in more detail.

Mature pollen from white and colored anthers was germinated on solidified medium according to Bino et al. (1987). Most of the transgenic white pollen grains had a collapsed phenotype, and neither these aberrant nor the healthy looking grains were able to form pollen tubes (Figure 4H), in contrast with transgenic purple pollen grains (Figure 4G).

DNA Gel Blot Analysis of the White Pollen F_1 Progeny Reveals Complete Linkage of the White Pollen Phenotype with One Antisense *chs* Gene Insert

To determine whether the white pollen phenotype is a stably inherited trait, a male sterile transformant with white pollen containing one T-DNA insert was crossed with petunia (V30). Thirteen of 30 F_1 plants inherited the white pollen phenotype. In 12 of the 30 F_1 plants, the presence of an antisense *chs* gene was analyzed by DNA gel blotting. To light up only the T-DNA insert, neomycin phosphotransferase II (NPTII) DNA was used as a probe. Figure 6 shows that the antisense phenotype and the presence of the T-DNA insert completely cosegregate. The introduced gene is present in the genomic DNA of the analyzed F_1 plants with white anthers (lanes 2 to 5, 7, and 9) and absent in the genome of F_1 plants with purple-colored anthers (lanes 1, 6, 8, and 10 to 12). We conclude that in the F_1 population analyzed, there is a complete linkage of the white pollen phenotype with one antisense *chs* gene insert.

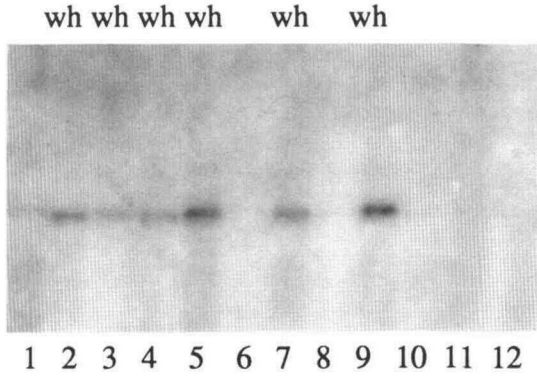

Figure 6. Linkage of White Anthers and the Presence of a CaMV 35S/Anther Box–Antisense *chs* cDNA Copy.

A CaMV 35S/anther box–antisense *chs* cDNA transformant (pTS20.4; containing one insert) was pollinated with petunia (V30), and the presence of a T-DNA insert in the progeny was analyzed by DNA gel blotting using the coding sequence of NPTII as a probe. Genomic HindIII-digested DNA of F_1 plants carrying white pollen was run in lanes 2 to 5, 7, and 9 (wh), and DNA of plants with purple pollen was run in lanes 1, 6, 8, and 10 to 12 (unlabeled).

DISCUSSION

In this study, we demonstrated that the cell-type specificity of the purported constitutive CaMV 35S promoter can be altered by insertion of the *chs* anther box. This modified promoter was able to direct antisense *chs* gene expression in tapetum cells and, as a consequence, effectively down regulated pigmentation in anthers of transgenic plants. This led to a block in pollen development that resulted in male sterility.

The anther box has been described previously as a homologous sequence found within the promoters of petunia flavonoid genes that are active during an early stage in anther development (van Tunen et al., 1989). Promoter deletion analysis revealed, however, that the presence of this anther box within the *chs* promoter is not a prerequisite for anther-specific gene expression, suggesting that additional sequences within the minimal promoter may direct the organ-specific expression (van der Meer et al., 1990).

Plegt and Bino (1989) previously showed that the CaMV 35S promoter drives expression in almost all cell types of the anther except for the tapetal and sporogenous tissues. We confirmed this result (Figure 4) and showed that introduction of the *chsA* anther box sequence in the CaMV 35S promoter altered this expression pattern: the tapetal cell layer showed GUS activity superimposed on the basic CaMV–*gus* staining pattern. Although the anther box sequence used originates from the *chsA* promoter and the staining pattern in anthers of *chsA–gus* transformed plants discloses GUS activity in the outer parenchymatic cells of the connectivum and the tapetal cell layer (Koes et al., 1990), we cannot conclude that the anther box on its own directs expression in the tapetum cells. Modules that confer organ specificity to the *chs* or CaMV 35S promoter may act in concert with the anther box.

The approach to inserting boxes into a heterologous promoter to confer new specificities is not novel. Insertion of the CaMV 35S activation sequence factor-1 binding site into the pea ribulose bisphosphate carboxylase small subunit 3A promoter resulted in high expression driven by this chimeric promoter in roots (Lam et al., 1989). Recently, Weisshaar et al. (1991) have shown that light inducibility can be conferred to the truncated CaMV 35S promoter by fusing one to four copies of box I and box II of the parsley *chs* promoter in both orientations 5′ to the deleted CaMV 35S promoter. Furthermore, Skriver et al. (1991) have shown that short sequences from

the promoters of gibberellic acid– and abscisic acid–inducible genes, when inserted into the EcoRV site at −90, confer gibberellic acid and abscisic acid inducibility to the CaMV 35S promoter.

Previously, we have shown that the expression of an antisense *chs*A cDNA driven by either the CaMV 35S promoter or the *chs*A promoter can result in a severe reduction in flower pigmentation (van der Krol et al., 1988, 1990b). In these experiments, reduction of floral pigmentation was frequently observed in the tube and corolla, but the color of the anthers and pollen was never affected. A plausible explanation of this result can be inferred from the data of Plegt and Bino (1989), who showed that the CaMV 35S promoter is not active in the tapetum cells where the *chs*A promoter is most active and which is the principal site of pigment biosynthesis and nourishment of the sporogenic cells (Kehrel and Wiermann, 1985; Beerhues et al., 1989). The failure of the antisense *chs* gene driven by the *chs*A promoter to inhibit pigmentation of anthers could be due to the low expression controlled by this promoter in transgenic plants (van der Meer et al., 1990). Because the insertion of the anther box in the CaMV 35S promoter added a tapetum cell layer specificity to the high expression of the basal CaMV 35S promoter, this chimeric promoter was used to express antisense *chs* sequences. Five of 35 plants showed a clear reduction of anther and pollen pigmentation, which confirms the involvement of the anther box in tapetum-specific expression.

The experiments presented in this paper indicated that transcription of an antisense gene in the appropriate cell type is a prerequisite to obtaining phenotypic effects. Although this result seems straightforward, previous experiments failed to reveal the presence of antisense *chs* RNA in tissue that showed the antisense phenotypic effects (van der Krol et al., 1990a, 1990b). The results also indicated that the copy number and orientation of the inserted anther box affect the antisense phenotype neither in a quantitative nor a qualitative way. A larger number of transformants will have to be analyzed, however, to prove this point. In addition, the position of the anther box (at −243 in the original *chs* promoter and at −90 in the CaMV 35S promoter) does not seem to be essential for correct regulation of gene expression (see also Lam et al., 1989; Skriver et al., 1991; Weisshaar et al., 1991). Furthermore, there was no correlation between reduction of pigmentation in anthers and corolla; all four combinations were found (Figure 4F), probably due to so-called position effects. DNA gel blot analysis of progeny segregating for white pollen revealed complete linkage of the antisense phenotype with the presence of a T-DNA copy (Figure 6).

Transformed plants carrying white pollen were male sterile due to a defect in pollen development (Figure 4H). This result strongly points to a hitherto undescribed function for flavonoids during male gametogenesis, although formally we cannot exclude the possibility that accumulation of precursors such as coumaric acid or caffeic acid may inhibit pollen development. We consider it rather unlikely, however, that a twofold to threefold overproduction of these precursors will be toxic to the gametophyte. Moreover, these compounds only displayed toxicity in the in vitro pollen maturation system when applied at the millimolar range (see below).

So far, two other cases of male sterility have been associated with white pollen. First, Coe et al. (1981) analyzed a white pollen mutant in maize (*whp*) that turned out to be male sterile. White pollen is determined by the double recessive condition for the *chs* gene (*C2*) with a factor *whp*. Only recently, Franken et al. (1991) showed that *whp* represents a second *chs* locus that is only expressed in the male reproductive tissue. Second, in studying cosuppression of *chs* genes using the doubly enhanced CaMV 35S promoter (Jorgensen, 1990), plants were recovered occasionally that showed white anthers containing white pollen. In this case, the pollen looked normal but failed to germinate in vitro. Self-pollination failed to induce seed set. The defect could be overcome by exogenous application of flavonoids both in vitro and in vivo, resulting in germination and seed set, respectively. Altogether this shows that in these transgenic petunias, the requirement for flavonoids in pollen can be satisfied at germination (Taylor and Jorgensen, 1992). In contrast to the situation found in maize and petunia, *chs* mutants in Arabidopsis and snapdragon are known to be male fertile. For both of the mutants of Arabidopsis (TT4) and snapdragon (*nivea*), however, it is not known whether *chs* gene expression is also affected in anthers. A functional *chs* gene copy may still be active in the anthers of such mutants. Alternatively, the presence of flavonoids in developing anthers may not be a prerequisite in all plant species. This point is under investigation.

Recent data indicated that flavonoids also have an additional function at a later stage in the reproductive cycle. A positive effect of flavonols on germination frequency and pollen tube length was observed when these compounds were added to the germination medium of petunia and tobacco pollen that maturated in vitro (B. Ylstra, O. Vincente, and E. Heberle-Bors, unpublished results). Future experiments will focus on the analysis of the function of flavonoids during gametophyte development and restoration of male fertility.

Recently, nuclear male sterility was engineered in higher plants by expressing fungal ribonucleases in tapetum cells, leading to the destruction of the tapetum and thereby to a premature release of unripe precursors (Mariani et al., 1990). This is in contrast with the approach we used in which the synthesis of a natural compound vital in pollen development is blocked by genetic engineering.

METHODS

DNA Methodology

DNA isolation, subcloning, restriction analysis, and sequencing were performed using standard procedures (Maniatis et al., 1982). Isolation of DNA from individual petunia transformants and DNA gel blot analysis were performed as described previously (Koes et al., 1987).

Construction of Chimeric Genes

The chimeric β-glucuronidase (*gus*) constructs were generated by cloning the *gus* coding region of pRAJ275 (Jefferson et al., 1987) as a HindIII-EcoRI fragment (filled in with the Klenow fragment of DNA polymerase I) into the SmaI site of VIP122 that contains the chalcone synthase A gene (*chs*A) 3′ untranslated region (van der Meer et al., 1990). This construct was cloned into the plasmid pTZ18R (Promega), yielding pTS19. The cauliflower mosaic virus (CaMV) 35S promoter of VIP102 (van der Krol et al., 1988) digested with EcoRI and BamHI was inserted into pTS19 cut with EcoRI and BamHI, resulting in pTS23 (see also Figure 2).

The oligonucleotides 5′-GAGCTCTAGAGGTGACAGAAATCTGCAG-3′ and 5′-CTGCAGATTTCTGTCACCTCTAGAGCTC-3′ were annealed, 5′ phosphorylated using T4 kinase, and cloned into pTS23 digested with EcoRV, yielding pTS24. The synthetic anther box was also cloned into the EcoRV site of the CaMV 35S promoter of VIP102 (CaMV–antisense *chs*A; van der Krol et al., 1988). The orientation and approximate copy number of the inserted anther boxes were determined by end labeling the BamHI restriction site 100 bp downstream of the anther box insertion followed by partial digestion of one of the restriction sites flanking the anther box. The end-labeled fragments were run on a sequencing gel. The orientation and the exact number of cloned anther boxes were determined by sequence analysis. All chimeric genes contained perfect copies of the anther box. The following antisense constructs were introduced in petunia plants: pTS20 (with a single anther box in the normal orientation), pTS21 (with two copies of the anther box in the reverse orientation), and pTS22 (with eight copies of the anther box in the normal orientation). All chimeric *gus* and antisense constructs were inserted as EcoRI-HindIII fragments into the binary vector Bin 19 (Bevan, 1984).

Plant Transformation

The constructs were transferred to *Agrobacterium tumefaciens* (LBA4404) by triparental mating. Exconjugants were used to transform *Petunia hybrida* leaf explants as described by Horsch et al. (1985). Leaf discs were prepared from top leaves of young, nonflowering plants. Chimeric *gus* genes were transferred to *P. hybrida* var W115, whereas antisense *chs* genes were transferred to the purple-flowering petunia VR hybrid. The flowers of this hybrid carry purple anthers and pollen. After shoot and root induction on kanamycin-containing media, plants were put in soil and transferred to the greenhouse. Plants regenerated (on media without kanamycin) from leaf discs treated with the LBA4404 strain lacking a binary vector served as controls. In total, 84 independent transformants were regenerated (49 transgenic plants for the CaMV–*gus* constructs and 35 transgenic plants transformed with the antisense *chs* genes). To determine the number of inserted T-DNA copies of some of them, DNA was extracted from leaves of individual transformants and subjected to DNA gel blot analysis using the neomycin phosphotransferase II (NPTII) coding sequence as a probe. The number of inserts varied from one to seven as deduced by the number of hybridizing border fragments (data not shown).

Fertile primary transformants were self-pollinated, and seeds were collected. The male sterile transformants with white anthers were cross-pollinated with petunia variety V30.

GUS Extractions and Fluorometric and Histochemical GUS Assays

Fresh material was collected from the transgenic plants and used for the GUS assays. GUS extractions and fluorometric GUS activity measurements were performed according to Jefferson et al. (1987). Fluorescence values were corrected for quenching of the extract by measuring the increase in fluorescence after the addition of a known amount of 4-methylumbelliferyl. Protein concentrations were determined using the Bio-Rad protein assay with BSA as a standard.

Histochemical localization of GUS activity was performed essentially as in Koes et al. (1990). Before staining, anthers were cut in two with a razor blade. To eliminate background GUS activity in anthers, X-gluc staining solution was adjusted to pH 8.0. If the pH of the staining buffer was reduced to 7.0, background staining was observed in untransformed anther tissue; therefore, all staining was performed at pH 8.0. 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 anthers of transgenic and untransformed plants. For analysis at the single cell level, X-gluc–stained tissues were fixed and embedded in paraffin according to Koes et al. (1990). Using a microtome, 7-μm-thick sections were cut and examined by bright-field microscopy after removing the paraffin.

Detection of Flavonoids

Anthers of 10 buds were incubated in 1 mL of 2 M HCl for 16 hr, and after hydrolysis (20 min at 100°C), flavonoids were extracted in a small volume of isoamyl alcohol and separated on cellulose thin-layer chromatography plates (Merck) using acetic acid/hydrochloric acid/water (30:3:10) as eluant. Standards of different precursors of flavonoid biosynthesis were used in the analysis.

In Vitro Pollen Germination

Plants were grown at 18 to 22°C under standard greenhouse conditions. Pollen was collected from flowers at anthesis and germinated on solidified medium containing 3 mM H_3BO_3, 1.7 mM $Ca(NO_3)_2$, 10% sucrose, 0.7% agar, pH 5.8, as described by Bino et al. (1987).

Isolation of RNA and RNase Protection

Anthers of five to seven buds (10 to 20 mm) were used for isolation of RNA (Koes et al., 1989). Endogenous *chs* mRNA was detected by RNase protection assays, performed according to van Tunen et al. (1988), using a full-sized *chs*A cDNA cloned in pTZ18U as a probe (Koes et al., 1989).

Isolation of DNA and DNA Gel Blot Analysis

Plant DNA was isolated from leaf tissue as described by Koes et al. (1987). DNA gel blot analysis was performed according to Koes et al. (1987), in which an NPTII fragment was used as a probe.

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