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, 1986) 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 Kroel 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 Kroel et al., 1988) or by the homologous chs promoter (van der Kroel 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
To achieve antisense inhibition of anther pigmentation, cell-type specific expression of the strong CaMV 35s promoter of the gus gene in anthers is diffuse and that the tapetum cells (1989) showed that the CaMV 35s-driven expression pattern to -221) at position -90 into the EcoRV site. 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 (chsA, chsJ: Koes et al., 1989; chalcone flavanone 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). To determine whether the insertion of the anther box into the CaMV 35S promoter alters its spatial expression, GUS expression in anther tissue and pollen of transgenic petunia plants. From top to bottom: synthetic chsA anther box sequence used in this study; anther box sequence found in the promoters of chsA, chb, chsJ, and dfrA, respectively. Capital letters represent nucleotides homologous to the sequence of the chb 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 versus 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 parenchymal cells of the connectivum, four loculi containing the sporogenic tissue (pollen), and the endothecium. Of the loculi is surrounded 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.
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).

Insertion of the Anther Box in the CaMV 35S Promoter Driving Antisense chs Genes

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 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/ancher box-gus transformants (24 plants; hatched bar). The ratios differ significantly, as judged from the Wilcoxon's rank test (a < 0.05).
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

<table>
<thead>
<tr>
<th>Corolla/Anthers</th>
<th>Purple/ Purple</th>
<th>Purple/ White</th>
<th>White/ Purple</th>
<th>White/ White</th>
<th>Total</th>
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<tbody>
<tr>
<td>pTS20</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>pTS21</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>pTS22</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td></td>
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</tbody>
</table>

* Total number of transformants.

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).

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

Figure 5. RNase Protection Analysis of Endogenous chs mRNA in Anthers and Corolla of pTS20 (CaMV/Anter 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 cDNA RNA results in four subfragments (see text).
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.

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 downregulated 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).

Piege 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 parenchymatous 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 chsA cDNA driven by either the CaMV 35S promoter or the chsA 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 chsA 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 chsA 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).
The constructs were transferred to Agrobacterium tumefaciens with the antisense chs genes. To determine the number of inserted T-DNA copies of some of them, DNA was extracted from leaves of independent 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).

Chimeric discs were prepared from top leaves of young, nonflowering plants. Anthers of 10 buds were incubated in 1 mL of 2 M HCl for 16 hr, and anthers were cut and examined by bright-field microscopy after removing the paraffin. 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 before 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 isooamyl 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 H3BO3, 1.7 mM Ca(NO3)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 chsA cDNA cloned in pZIRU 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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