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RESEARCH ARTICLE

Regulatory Genes Controlling Anthocyanin Pigmentation Are Functionally Conserved among Plant Species and Have Distinct Sets of Target Genes

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In this study, we demonstrate that in petunia at least four regulatory genes (*anthocyanin-1* [*an1*], *an2*, *an4*, and *an11*) control transcription of a subset of structural genes from the anthocyanin pathway by using a combination of RNA gel blot analysis, transcription run-on assays, and transient expression assays. *an2*$^-$ and *an11*$^-$ mutants could be transiently complemented by the maize regulatory genes *Leaf color* (*Lc*) or *Colorless-1* (*C1*), respectively, whereas *an1*$^-$ mutants only by *Lc* and *C1* together. In addition, the combination of *Lc* and *C1* induces pigment accumulation in young leaves. This indicates that *Lc* and *C1* are both necessary and sufficient to produce pigmentation in leaf cells. Regulatory pigmentation genes in maize and petunia control different sets of structural genes. The maize *Lc* and *C1* genes expressed in petunia differentially activate the promoters of the chalcone synthase genes *chsA* and *chsJ* in the same way that the homologous petunia genes do. This suggests that the regulatory proteins in both species are functionally similar and that the choice of target genes is determined by their promoter sequences. We present an evolutionary model that explains the differences in regulation of pigmentation pathways of maize, petunia, and snapdragon.

INTRODUCTION

Flavonoids constitute a family of secondary metabolites common to all higher plants. They are synthesized by way of a branched pathway yielding different subclasses of flavonoids, as shown in Figure 1, each serving a variety of functions in plant development and reproduction (for review, see van der Meer et al., 1992c; Martin and Gerats, 1993). Genes encoding flavonoid enzymes have been isolated from a variety of plant species. In most plant systems analyzed, flavonoid biosynthetic genes are coordinately and tissue specifically expressed (reviewed by van der Meer et al., 1992c). Various patterns of expression are seen among plant species, which is in line with the observed variability in accumulation patterns and biological functions of flavonoids.

In petunia flowers, for example, a coordinated transcriptional activation of chalcone synthase (*chs*), chalcone flavanone isomerase (*chi*), and dihydroflavonol 4-reductase (*dfr*) genes occurs in the young petals and anthers, resulting in the accumulation of both colored anthocyanins and colorless flavonols (van Tunen et al., 1988, 1990; Beld et al., 1989; Koes et al., 1990). In the ovary, *chs* and *chi* genes are transcriptionally activated in ovules, but *dfr* genes are activated only slightly, resulting in the accumulation of flavonols only (Koes et al., 1990; van Tunen et al., 1990; H.S.M. Huits, M.M. Kreike, J.N.M. Mol, A.G.M. Gerats, and R.E. Koes, unpublished results). In seeds, *chs*, *chi*, and *dfr* genes are activated in the pericarp, resulting in the synthesis of an as yet undefined class of flavonoids, most likely proanthocyanidins (Koes et al., 1990). Developing maize seeds express structural flavonoid genes in the aleurone cell layer of the endosperm and the scutellum of the embryo, resulting in the synthesis of anthocyanins in both tissues (Tonelli et al., 1991; Consonni et al., 1993). In mature maize plants, structural genes can be activated in a variety of tissues, where they are responsible for the anthocyanin pigmentation of leaves, stems, anthers, and kernels (Radicella et al., 1992).

In addition, the expression of maize and petunia flavonoid biosynthetic genes is subject to a different control by phytohormones. The transcriptional activation of *chs* genes in the flower corolla of petunia is dependent on the phytohormone gibberellic acid (Weiss et al., 1990, 1992), whereas the phytohormone abscisic acid is involved in the control of pigmentation of the maize aleurone layer (Hattori et al., 1992). The observed variable accumulation patterns of flavonoids and their very different functions raise the questions of how expression of flavonoid biosynthetic genes is regulated and how their expression patterns have been established during evolution.

Genes controlling the expression of flavonoid genes have been identified by mutations in three plant species: maize,

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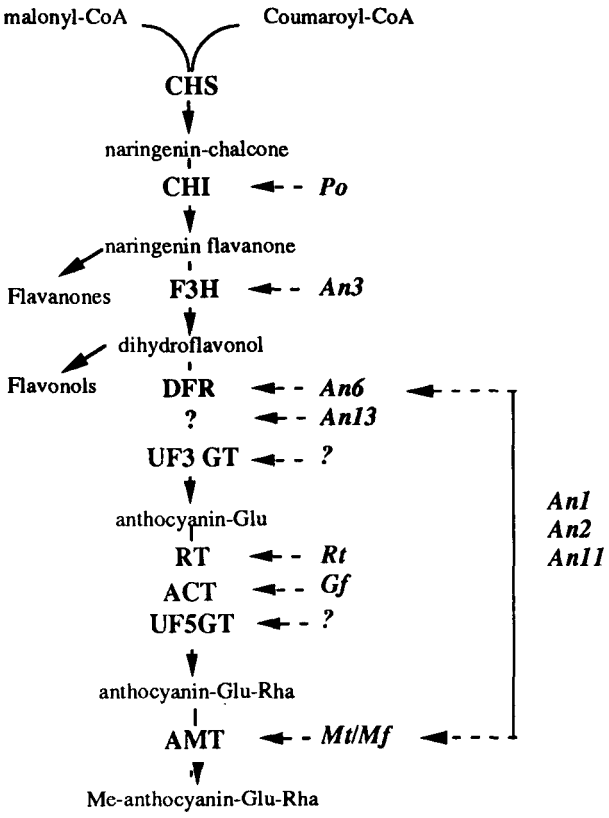

Figure 1. Schematic Representation of the Flavonoid Biosynthetic Pathway.

Abbreviations are as follows: CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; UF3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; RT, anthocyanin rhamnosyltransferase; ACT, anthocyanin acyltransferase; UF5GT, UDP-glucose:flavonoid 5-*O*-glucosyltransferase; AMT, anthocyanin methyltransferase. Genetic loci controlling single enzyme activities are represented in italics: *Po*, *Pollen*; *An3*, *Anthocyanin-3*, *An6*, *Anthocyanin-6*; *An13*, *Anthocyanin-13*; *Rt*, *Rhamnosylation at three*; *Gf*, *Glucosylation at five*; *Mt*, *Methylation at three*; *Mf*, *Methylation at five*. The *an13* gene product is involved in one of the reactions that convert a dihydroflavonol into an anthocyanin glucoside, but the exact biochemical function is unknown, as indicated by the question mark. The loci *An1*, *An2*, and *An11* control the activity of multiple enzymes and are indicated separately.

snapdragon, and petunia. In maize, two families of regulatory genes have been identified, the *R/B* and *C1/Purple* (*Pl*) families, whose gene products are required for the expression of several structural flavonoid genes (for a review, see Dooner et al., 1991). Both the *Colorless-1* (*C1*) and *R* types of regulatory genes were cloned by transposon tagging (Cone et al., 1986; Paz-Ares et al., 1987; Dellaporta et al., 1988). The C1 protein shares homology with the DNA binding domain from the animal transcription activator c-*myb* (Cone et al., 1986; Paz-Ares et al., 1987). Proteins encoded by the genes *Leaf color* (*Lc*), *B*, and *Sienna* (*Sn*)—all members of the *R* gene family—contain a basic helix-loop-helix (bHLH) domain, similar to that found in mammalian transcription activators (Ludwig et al., 1989; Perrot and Cone, 1989; Consonni et al., 1993). In transient expression systems, the combined expression of *C1* and one of the *R* genes activates the promoter of structural flavonoid genes, providing direct evidence for the role of *C1* and *R* genes in transcriptional activation (Goff et al., 1990, 1991; Roth et al., 1991; Bodeau and Walbot 1992). Recently, Lloyd et al. (1992) showed that the stable introduction of an *Lc* gene in tobacco and Arabidopsis can intensify pigmentation in several tissues, indicating that pigmentation genes from species other than maize can also be activated by *Lc*.

In snapdragon, mutations in the genes *delila* and *eluta* result in a drastic reduction in expression of the genes encoding the enzymes flavanone 3-hydroxylase (F3H), UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UF3GT), and DFR (Almeida et al., 1989; Martin et al., 1991). The *delila* gene was recently cloned via transposon tagging (Goodrich et al., 1992). It encodes a protein homologous to *Lc*, suggesting that *delila* also acts as a transcription activator.

In petunia, five genetically defined genes (*anthocyanin-1* [*an1*], *an2*, *an6*, *an10*, and *an11*) control the steady state level of *dfr* mRNA in the limb of the petal (Beld et al., 1989). Restriction fragment length polymorphism mapping and complementation studies showed that the *an6* and *dfrA* genes are identical (H.S.M. Huits, M.M. Kreike, J.N.M. Mol, A.G.M. Gerats, and R.E. Koes, unpublished data); therefore, the other four genes, *an1*, *an2*, *an10*, and *an11*, are likely to act in *trans* on *dfr* expression. The enzyme activities of UF3GT, UDP-glucose: flavonoid 5-*O*-glucosyltransferase (UF5GT), and the anthocyanin methyltransferases (AMTs) are also reduced in *an1*$^-$ and *an2*$^-$ flowers (Gerats et al., 1984; Jonsson et al., 1984; Beld et al., 1989), further supporting the idea that these loci have a regulatory function.

In this study, we present an analysis of the regulatory pigmentation genes from petunia. In addition to the previously described regulatory genes *an1*, *an2*, *an10*, and *an11* (Beld et al., 1989), we identified another regulatory gene, *an4*, that regulates expression of anthocyanin biosynthetic genes in the anthers. We show that the *an1*, *an2*, and *an11* gene products activate the promoters of flavonoid biosynthetic genes and that mutations in these three regulatory pigmentation genes can be complemented by the maize factors *Lc* and *C1*. This indicates that regulatory pigmentation genes in petunia and maize are functionally similar, despite the wide evolutionary divergence and the extreme differences in pattern and function of pigmentation of maize and petunia.

RESULTS

an1, *an2*, *an4*, and *an11* Genes Affect the Steady State mRNA Level of At Least Five Genes

Wild-type petunia plants accumulate purple- or red-colored anthocyanins in the limb and tube of the flower corolla, the

anthers, and the stem just beneath the flower. Seeds accumulate anthocyanin derivatives (presumably proanthocyanidins) in the seed coat (Koes et al., 1990). A number of mutations that reduce or fully block pigmentation in one or more of these tissues have been identified, as depicted in Table 1. Accumulation of dihydroflavonols has been shown for some of these mutants (Gerats et al., 1982). Beld et al. (1989) reported that mutations in *an1*, *an2*, *an6*, *an10*, or *an11* each result in an absence of *dfr* mRNA in the corolla limb, whereas *chs* mRNA is still detectable. Both observations suggest a division of the pathway into two independently regulated units.

To investigate directly whether a regulatory division in the flavonoid pathway occurs in petunia, we measured the steady state mRNA levels of phenylalanine ammonia-lyase (*pal*), *chs*, *chi*, *f3h*, *dfr*, *an13*, *rhamnosylation at three* (*rt*), and *amt* in the corolla limb of a large number of wild-type and mutant petunia lines. The function of the enzymes encoded by these genes is shown in Figure 1. Among the selected petunia lines were the wild-type progenitor, transposon insertion mutants, wild-type revertant derivatives, and stable recessive derivatives for the genes *an1*, *an2*, and *an11* (Table 1; see Methods for details). Because the accumulation of flavonoid mRNAs is regulated during development of the flower, we used flowers of three different developmental stages for each line. In Figure 2, we show the results for a subset of the lines that were used.

Flower limbs of the line V26 (*an4*$^-$) accumulated all tested flavonoid mRNAs (Figure 2) to levels that were similar to those in wild-type lines (VR and V30; data not shown). This was expected, because V26 has only a mutation in the anther-specific gene *an4* and is wild type for all other anthocyanin genes. The *pal* probe detected two transcripts that appeared to accumulate differentially (Figure 2A). In RNA extracted from leaves, only the short *pal* transcript was detected (data not shown). It is possible that the two *pal* transcripts originate from two differentially expressed members of a *pal* gene family, as has been described for bean (Cramer et al., 1989; Liang et al., 1989).

In flower limbs of the *an6*$^-$ line W80, no *dfr* mRNA could be detected, whereas the accumulation of all other flavonoid mRNAs is comparable to that in V26. This was not unexpected, because the *An6* locus harbors one of the three *dfr* genes (designated *dfrA*) and implies that all *dfr* mRNA in the flower limb is derived from this single gene (Beld et al., 1989; H.S.M. Huits, M.M. Kreike, R.E. Koes, J.N.M. Mol, and A.G.M. Gerats, unpublished data). In flower limbs of the *an2*$^-$ mutant W115, *dfr*, *an13*, *rt*, and *amt* mRNAs are strongly reduced compared to V26. Accumulation of the same mRNAs was also reduced in flower limbs of W82 (*an2*-mutable) compared to isogenic W82 revertant plants. The low amounts of *dfr*, *an13*, *rt*, and *amt* mRNAs detected in the mutable W82 plants may result from the leaky nature of this mutant allele and from somatic reversion events (the spots in the flower). In flower limbs from the *an1*$^-$ and *an11*$^-$ lines W162 and W134 (Figure 2), as well as the *an1*$^-$-mutable line W138 (data not shown), a strong reduction of *dfr*, *an13*, *rt*, and *amt* mRNAs was observed when compared to the corresponding wild-type line (R27). W134 (*an11*$^-$) and W138 (*an1*-mutable) are isogenic to R27, whereas W162 is closely related but not fully isogenic to R27.

Mutations in *an1*, *an2*, and *an11* genes had at most a small effect on the quantitative accumulation of *pal*, *chs*, *chi*, and *f3h* mRNAs, which occurs early in the flavonoid pathway. Because we used an isogenic wild type for all three mutants, it can be excluded that the differences in hybridization were due to different genetic backgrounds. The small quantitative reductions may be due to an indirect effect of the *an1*$^-$, *an2*$^-$, and *an11*$^-$ mutations or, alternatively, to physiological differences between the different plants. In contrast to the results of Beld

Table 1. Genotypes and Phenotypes of the Different Petunia Lines Used in This Study

Line	Mutation[a]	Anthocyanin Accumulation[b]			
		Corolla Limb	Tube	Anthers	Seed Coat
V30	None	+	+	+	+
VR	None	+	+	+	+
V26	an4$^-$	+	+	–	+
R27	an4$^-$	+	–	–	–
W44	an2$^-$	–	–	+	+
W80	an4$^-$,an6$^-$	–	–	–	–
W82	an2-mut,an4$^-$	Spotted	+	–	+
W82 revertant	an4$^-$	+	+	–	+
W115	an2$^-$,an4$^-$	–	+	–	+
W134	an4$^-$,an11$^-$	–	–	–	–
W137	an4$^-$,an11-mut	Spotted	–	–	ND
W138	an1-mut,an4$^-$	Spotted	–	–	Spotted
W154	an10$^-$, an4$^-$	–	–	–	–
W162	an1$^-$,an4$^-$	–	–	–	–

[a] Only mutations relevant to this study are listed. For a further description, see de Vlaming et al. (1984). -mut denotes a mutable allele caused by insertion of a transposable element. Stable recessive alleles are denoted by a minus sign.

[b] +, anthocyanins present; –, anthocyanins absent or reduced; ND, not determined.

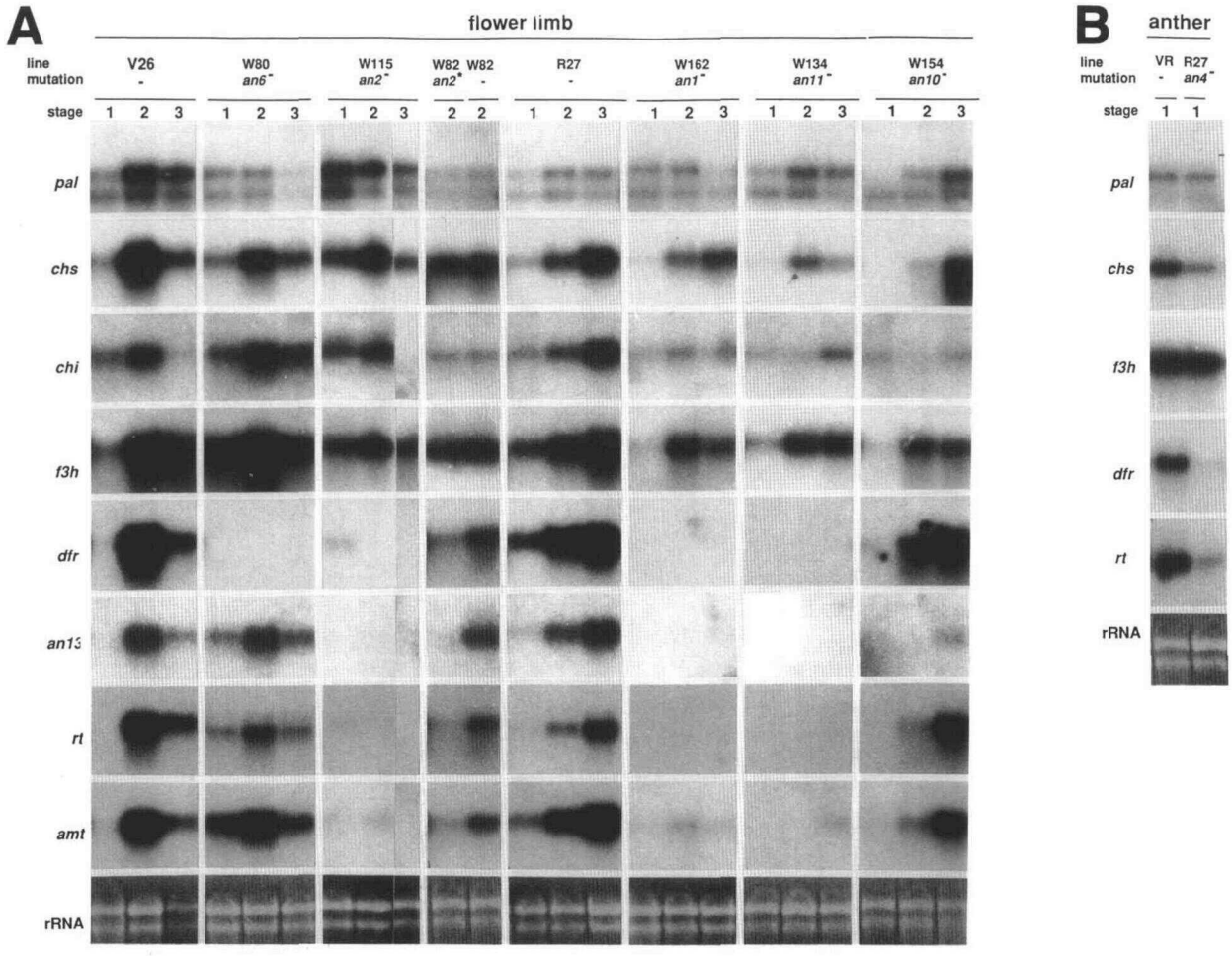

Figure 2. Analysis of the Steady State mRNA Levels of Flavonoid Genes in Different Petunia Lines.

(A) RNA gel blot analysis of total RNA (10 μg) extracted from the flower limb of various lines harvested at three developmental stages (1, 2, and 3). Mutations that affect pigmentation of the flower limb are indicated above. All lines are *an4*⁻. Because this does not affect pigmentation and gene expression in the flower limb, it is not further indicated. rRNA was visualized by ethidium bromide staining and served as a control for equal loading and integrity of the RNA. The flavonoid biosynthetic mRNAs were visualized by hybridization. Stages in flower development were defined according to the length of the bud (the length of a fully stretched but not yet opened bud was taken as 100%). Stage 1, 10 to 25%; stage 2, 25 to 50%; stage 3, 50 to 100% of maximal length.

(B) RNA gel blot analysis of total RNA from anthers (stage 2) of a wild-type (VR) and an *an4*⁻ petunia line (R27). rRNA was visualized by ethidium bromide staining and the flavonoid biosynthetic mRNAs were visualized by hybridization.

et al. (1989), we did not observe a dramatic effect of a mutation in the *an10* gene on the expression of any of the flavonoid genes analyzed (see Discussion).

To investigate the role of the *an4* gene in the pigmentation of anthers, we analyzed the expression of a set of structural genes from the pathway in anthers of an *an4*⁻ mutant line. Expression of flavonoid genes in anthers is limited to an early and well-defined stage in flower development (van Tunen et al., 1988; Koes et al., 1989a); therefore, we isolated anther RNA from this developmental stage only. Figure 2B shows that the mRNA levels of *dfr* and *rt* in *an4*⁻ anthers were strongly reduced compared to those in wild-type anthers, whereas *chs* and *f3h* mRNA levels in wild-type and *an4*⁻ anthers were similar. Therefore, we concluded that *an4* is a regulatory gene controlling the expression of multiple flavonoid biosynthetic genes in anthers.

Except for the single *f3h* gene (Britsch et al., 1991), all other analyzed genes (*pal*, *chs*, *chi*, *dfr*, *an13*, *rt*, and *amt*) comprise small gene families. From the gene families encoding CHI, DFR, AN13, and RT, only one member is active in corolla tissue (Kroon et al., 1993), implying that the *chi*, *f3h*, *dfr*, *an13*, and *rt* mRNAs detected in the RNA gel blot analysis were

transcribed from single genes. The *chs* gene family, however, contains two members (*chsA* and *chsJ*) that are transcriptionally active in the corolla limb (Koes et al., 1989a). Under the conditions employed in the RNA gel blot analysis (Figure 2), the *chs* probe detected both transcripts and thus measured the total amount of *chs* transcripts. To test whether the two *chs* genes are under the same genetic control, we performed gene-specific RNase protection assays on RNA from wild-type and mutant lines. Figure 3 shows that the expression of the two *chs* genes appears to be differentially influenced by mutations in *an1*, *an2*, *an4*, or *an11*. The *chsA* mRNA level is at most slightly down-regulated in the $an1^-$, $an2^-$, and $an11^-$ lines, whereas the *chsJ* mRNA level is completely controlled by *an1*, *an2*, and *an11* in the corolla and by *an4* in the anthers (Figure 3).

In summary, the expression analyses show that *an1*, *an2*, *an11*, and *an4* are regulatory genes that control the expression of some (at least five), but not all, structural genes from the flavonoid pathway.

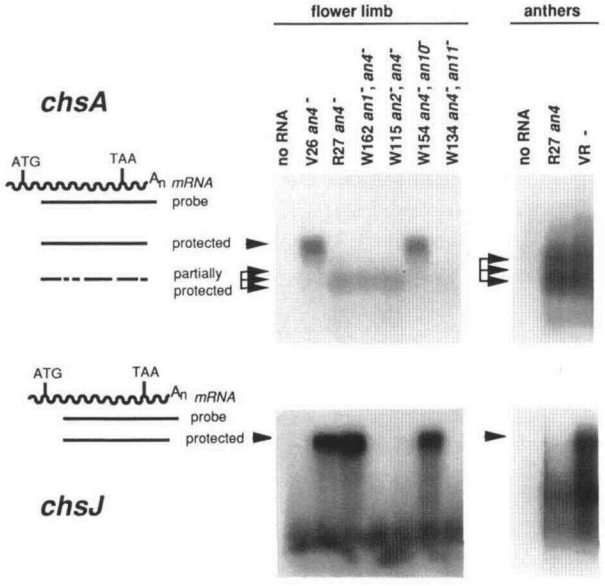

Figure 3. Expression of *chsA* and *chsJ* in Corollas and Anthers of Different Petunia Lines.

RNase protection of the *chsA* (top part) and *chsJ* probes (bottom part) by RNA (10 μg) extracted from flower limbs and anthers of various petunia lines. The diagrams on the left show the probes that were used and the protected fragments that were generated. The *chsA* probe is only partially protected by RNA from some petunia lines due to small sequence differences with the *chsA* gene of V30 from which the probe was prepared. As a consequence, multiple small protected fragments are generated (Koes et al., 1989a; van der Meer et al., 1992a), as is indicated in the diagram.

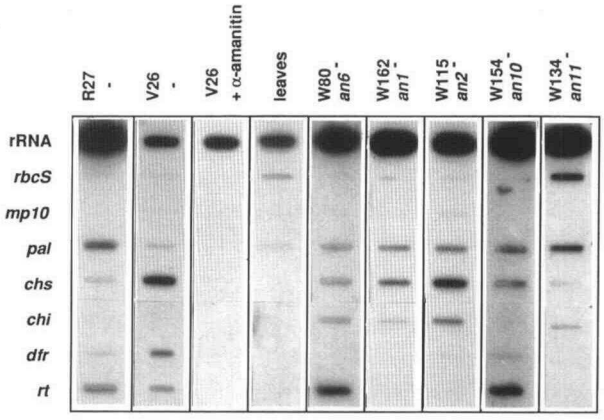

Figure 4. Analysis of the Transcription Initiation of *pal*, *chs*, *chi*, *dfr*, and *rt* in Different Petunia Mutants.

Nuclear run-on assays were performed utilizing nuclei of corollas (mixture of developmental stage 2 and 3) or leaves. Nascent ^{32}P-labeled RNAs were hybridized to filters carrying single-stranded DNA clones from the indicated flavonoid genes and control genes in the vector M13mp10. The empty vector, M13mp10, was loaded as a control to test for background hybridization. A sample of V26 corolla nuclei was exposed to α-amanitin (4 μg/L) to test for RNA polymerase II–specific transcript elongation.

Genes *an1*, *an2*, and *an11* Control Transcription of the *dfrA* and *rt* Gene in the Petal Limb

To examine whether the reduced steady state levels of *dfr* and *rt* mRNA observed in $an1^-$, $an2^-$, and $an11^-$ mutants are caused by a block in transcription or by a destabilization of the transcripts, we analyzed nascent RNA by a nuclear run-on assay. The levels of transcription from various flavonoid biosynthetic genes (*pal*, *chs*, *chi*, *dfr*, and *rt*) and control genes (encoding the small subunit of ribulose bisphosphate carboxylase [rbcS] and rRNA) were measured in nuclei isolated from the corolla limb of various wild-type and mutant lines. The results of these analyses are presented in Figure 4. In nuclei from corolla limbs of wild-type lines (V26 and R27), nascent RNA was detected for all the analyzed flavonoid genes. The transcription rates of the different flavonoid genes differ considerably in the same petunia line (e.g., transcription rates of *chs* genes versus those of *chi* genes). These differences are not always reflected in the steady state levels of the corresponding mRNAs (see Figure 2A), probably due to differences in mRNA stability. As expected, the rRNA genes are transcribed to a high extent in all tissues. We observed that the ratio between RNA polymerase I transcription (rRNA genes) and RNA polymerase II transcription (*rbcS* in leaves and the flavonoid genes in corolla) was variable among different preparations of nuclei from one petunia line. This may be caused by differences in stability of the RNA polymerases I and II.

Three independent lines of evidence demonstrate that the nascent transcripts detected in the nuclear run-on assays

correctly reflect the in vivo situation. First, transcription of flavonoid biosynthetic genes (but not that of rRNA genes) is inhibited by α-amanitin, confirming that their transcription is dependent on RNA polymerase II activity. Second, the *an6* mutation affects only *dfr* transcription, which is in agreement with the observation that the *dfrA* and the *an6* gene are identical. Third, no transcription of *chs*, *chi*, *dfr*, and *rt* genes could be detected in leaf tissue, whereas transcription of *rbcS* is still detectable.

Transcription run-on assays in nuclei prepared from *an1*$^-$, *an2*$^-$, or *an11*$^-$ lines showed that the transcription rates of the *dfr* and *rt* genes are drastically reduced, while the transcription rates of *chs*, *chi*, and *f3h* genes are comparable to those in wild-type lines. Therefore, we concluded that the products of the *an1*, *an2*, and *an11* genes control the transcription of the *dfrA* and *rt* genes.

Expression of a *dfrA*-β-Glucuronidase Chimeric Gene in the Petunia Corolla Is under the Control of the *an1*, *an2*, and *an11* Genes

To test whether *an1*, *an2*, and *an11* regulate transcription by interacting with *cis*-acting DNA elements in the promoter of their target genes, we constructed a chimeric gene consisting of the β-glucuronidase (*gus*) reporter gene (Jefferson et al., 1987) fused to the promoter of the *dfrA* gene, as shown in Figure 5, and studied its transient expression in mutant flowers using particle bombardment. In such experiments, the activity of the *dfrA* promoter in transformed cells is visualized as blue spots upon incubation of the tissues with a histochemical substrate for the GUS enzyme. The number of spots provides a measure of the level of promoter activity (Ludwig and Wessler, 1990; Bowen, 1992).

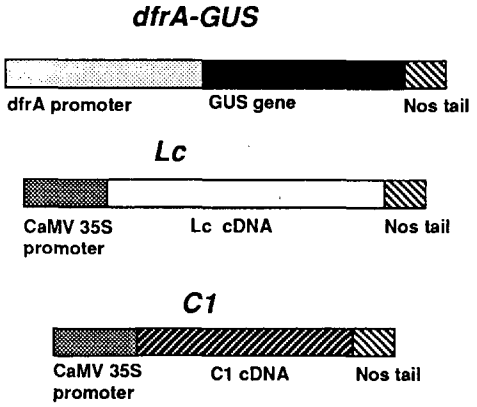

Figure 5. Schematic Representation of Effector and Reporter Genes Used in This Study.

A 2067-bp fragment of the 5′ flanking region of *dfrA* fused to the *gus* gene was used as a reporter gene to measure *dfrA* promoter activity. The regulatory proteins Lc and C1 were expressed by fusions of the corresponding cDNA behind the CaMV 35S promoter. (For further details, see Methods.)

Figure 6A shows that the *dfrA–gus* construct is active when introduced in corolla tissue of an *an6*$^-$ mutant line (W80). This was expected because the *an6* and the *dfrA* genes are identical. When a *gus* gene driven by the *chsA* promoter was introduced in corolla tissue from the same line, a comparable number of spots was observed (data not shown). In contrast, no blue spots were observed after transformation of the *dfrA–gus* fusion gene into *an1*$^-$, *an2*$^-$, or *an11*$^-$ corollas (Figures 6B, 6C, and 6E, respectively). When a *chsA–gus* gene was introduced in the same mutant lines, it resulted in a number of spots only slightly less than in the *an6*$^-$ line (data not shown). This is consistent with the RNA gel blot data (Figure 2A) and rules out the possibility that the failure of these mutant corollas to express the *dfrA–gus* gene is due to very low transformation rates. The *an10*$^-$ line behaved identically to the *an6*$^-$ line, again indicating that *an10* is not a regulatory gene (Figure 6D).

These results are fully consistent with the data obtained from the run-on analyses (Figure 4), and they indicate that mutations in the *an1*, *an2*, or *an11* genes prevent activation of the *dfrA* promoter, suggesting that they encode or regulate the activity of a transcription factor.

Stable Transformation of Petunia with the Cauliflower Mosaic Virus 35S-*Lc* Fusion Gene

The best-characterized transcription factors involved in the regulation of pigment formation in plants are those encoded by the *C1* and *R* gene families from maize. To examine whether similar factors are involved in transcriptional activation of petunia flavonoid genes, we tested whether these maize transcription factors can activate petunia flavonoid genes. We decided to express the *Lc* gene in petunia. A gene construct that consisted of the *Lc* protein coding sequence driven by the 35S promoter from cauliflower mosaic virus (CaMV) (Figure 5) was introduced into two petunia lines, V26 (*an4*$^-$) and W115 (*an2*$^-$ and *an4*$^-$). Both lines regenerate easily and are routinely used in transformation experiments.

In both genetic backgrounds (V26 and W115), ~80% of the transformed calli rapidly developed red/purple groups of cells, as shown in Figures 7A and 7B. None of these pigmented calli gave rise to shoots. From the calli that initially remained green, shoots regenerated, but most of these shoots turned purple in the very early stages of growth (Figure 7C) and did not produce roots or grow further. In contrast, green shoots were generally able to root. In one of the resulting plantlets, pigmentation of the young roots was observed (Figure 7D). In total, 15 W115 and 10 V26 transformants were obtained. No differences in pigmentation patterns were observed between plants transformed with the *Lc* gene fusion and control plants at this developmental stage. Gel blot analyses on RNA extracted from the leaves of the transformed plants indicated that only one of them accumulated a low amount of *Lc* mRNA (the plant in Figure 7D), whereas no *Lc* mRNA could be detected in the other transformants (results not shown). The single

Lc-expressing plant grew extremely slowly and produced only a few very aberrant flowers (data not shown).

The excessive pigment synthesis in *Lc*-transformed calli and regenerative tissue suggests that *Lc* can activate structural pigmentation genes from petunia. However, the apparent selection against *Lc*-expressing plants prevented us from testing whether a stably introduced *Lc* transgene could restore pigmentation in mutant flowers (also, see Discussion).

Regulatory Genes *C1* and *Lc* from the Maize Anthocyanin Pathway Can Activate the Petunia *dfrA* Promoter in Leaf Cells

As an alternative to analyses in transgenic plants, we turned to transient expression analyses. In these experiments, a reporter gene (*dfrA–gus*) was cointroduced with 35S–*Lc* and 35S–*C1* fusion genes (Figure 5) into various petunia tissues via particle bombardment.

In the first set of experiments, we introduced the *dfrA–gus* construct alone or together with the *Lc* and *C1* constructs in petunia leaves. In leaves bombarded with the *dfrA–gus* construct alone, very few cells expressing the gene were observed (Figure 6F). This suggests that the *dfrA* promoter is active at a very low level. In stably transformed plants, the *dfrA–gus* fusion gene is not expressed in leaves, but in some experiments its expression could be induced by wounding (F. Quattrocchio, J. Wing, and H.S.M. Huits, unpublished data). The low activity observed in the transient assays may therefore reflect wound-induced expression. When the *dfrA–gus* fusion gene was cointroduced with both the *C1* and *Lc* fusion genes, a dramatic increase in the number of blue spots was observed (Figures 6G and 6H), indicating that the *C1* and *Lc* genes are able to activate the *dfrA* promoter in *trans*. When the *dfrA–gus* fusion gene was cointroduced with either *Lc* or *C1*, the number of blue spots was comparable to those observed when leaves are bombarded with *dfrA–gus* alone (data not shown), implying that the activation of the *dfrA* promoter requires both *Lc* and *C1*.

When very young leaves were bombarded with *Lc*, *C1*, and the *dfrA–gus* gene, we often observed red spots that developed within 24 hr after bombardment (Figures 6H and 6I). Such red spots were not observed when the *dfrA–gus* gene was introduced alone. The presence of these colored cells indicated that the combined expression of *Lc* and *C1* not only activated the *dfrA* promoter, but also activated promoters of other genes involved in flavonoid biosynthesis.

Expression of *C1* or *Lc* either Alone or in Combination Complements Specific Regulatory *an* Mutations in Petunia

Because the combination of *Lc* and *C1* can activate promoters of structural pigmentation genes in petunia, similar factors may exist in petunia that control pigmentation in vivo. To test this, we studied reactivation of the *dfrA* promoter in the regulatory pigmentation mutants *an1*, *an2*, and *an11* by cointroduction with either *Lc* or *C1* via particle bombardment.

After cointroducing the *dfrA–gus* gene with the *Lc* and *C1* fusion genes, a large number of blue spots was observed in all three mutants, demonstrating that *Lc* and *C1* reactivate the *dfrA* promoter, as shown in Figures 6J to 6L and Table 2. To test whether *Lc*, *C1*, or the combination of both is responsible for this reactivation, we cointroduced the *dfrA–gus* gene with only one of the *Lc* or *C1* fusion genes. Reactivation of *dfrA–gus* in *an2*$^-$ petal limbs was observed when it was cobombarded with the *Lc* fusion gene (Figure 6N; Table 2) but not with the *C1* fusion gene (Figure 6Q; Table 2). Considering the smaller number of blue spots, *Lc* alone appears to activate the *dfrA* promoter less efficiently than the combination of *Lc* with *C1* (Table 2). The *Lc* gene was able to activate the *dfrA* promoter in *an1*$^-$ corolla tissue, albeit with an extremely low frequency, whereas no activation was observed in *an11*$^-$ corolla tissue (Figures 6M and 6O; Table 2). The *C1* fusion gene, however, was able to activate the cointroduced *dfrA–gus* gene in *an11*$^-$ corolla tissue (Figure 6R), but not in *an1*$^-$ (Figure 6P) or *an2*$^-$ (Figure 6Q) corolla tissue. The activation of the *dfrA–gus* gene in *an11*$^-$ tissue by *C1* alone was far less efficient than by the combination of *C1* plus *Lc* in the same tissues (Table 2).

The relatively efficient complementation of the *an2* mutation by the *Lc* fusion gene indicates that *an2* encodes or regulates a functionally similar transcription factor. Considering that *an2* only affects pigmentation of the limb of the corolla, we reasoned that another gene may encode a very similar factor that activates flavonoid genes in anthers. A good candidate to encode such a factor is the *an4* gene, which is involved in transcriptional activation of flavonoid genes in anthers (Figures 2 and 3 and Discussion). Although the high background GUS activity in pollen grains of petunia often obscured the activity of an introduced *dfrA–gus* gene (e.g., Figures 6A and 6P), we could occasionally observe blue spots in *an4* anthers after bombardment of the *dfrA–gus* gene together with the *Lc* gene when the anthers were only briefly stained for GUS activity (Figure 6T). Such spots were not observed when the *dfrA–gus* gene was introduced without the *Lc* gene (Figure 6S).

In summary, the complementation experiments indicated that the *an2* and *an4* genes encode or control a transcription factor that is functionally homologous to *Lc*, whereas *an11* may encode or control a factor functionally related to *C1*. The role of the *an1* gene, however, remains unclear.

Differential Induction of the *chsA* and *chsJ* Promoters by Lc and C1

Despite the conservation of function between regulatory pigmentation genes from maize and petunia, there is a striking difference with regard to the sets of target genes that are controlled by these regulators. In maize, the *Lc* and *C1* genes control expression of all the anthocyanin biosynthetic genes (Dooner et al., 1991), whereas the *an1*, *an2*, *an4*, and *an11*

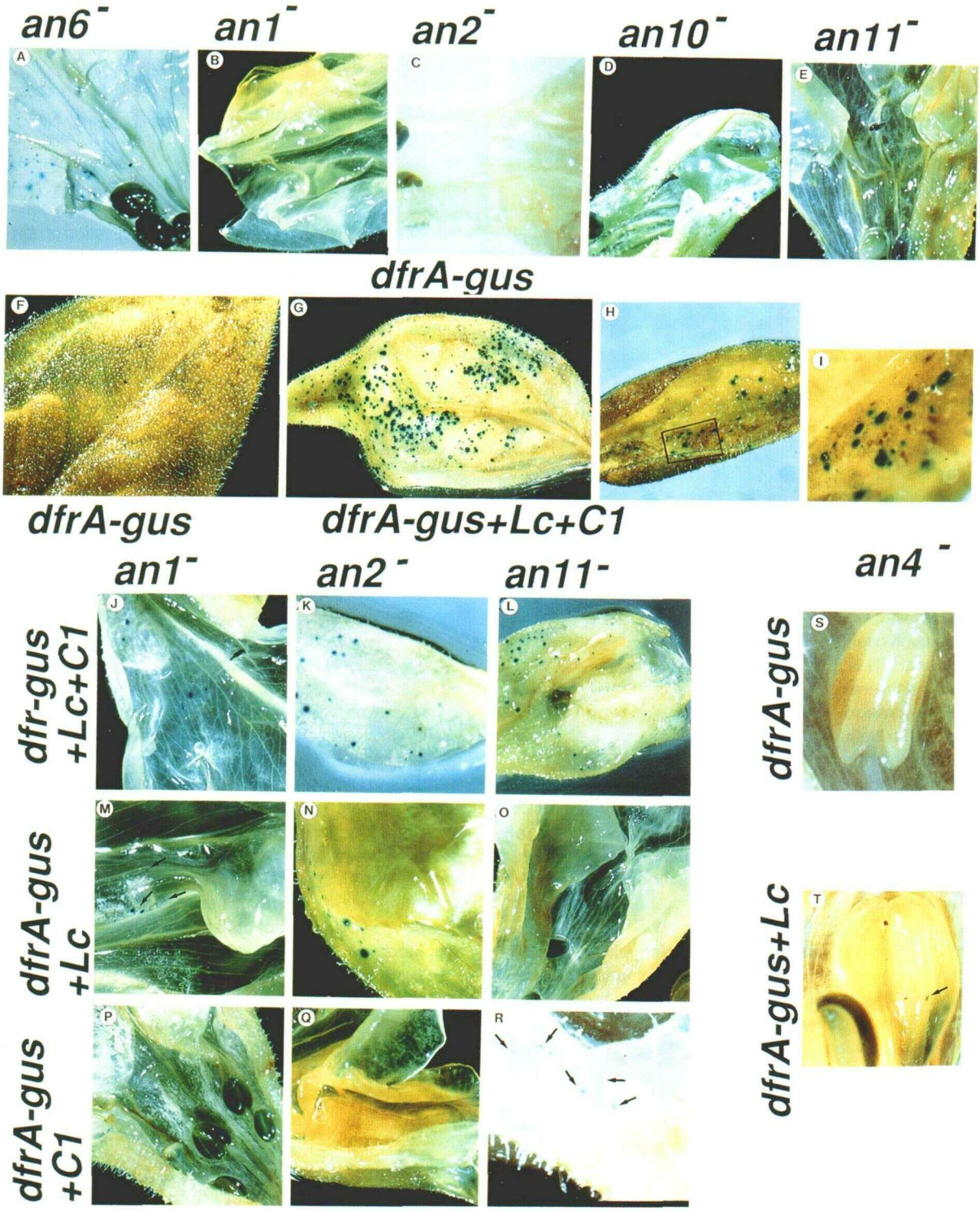

Figure 6. Transient Expression Analyses of the *dfrA–gus* Gene in Different Petunia Mutants.

Transient expression of a *dfrA–gus* reporter gene was measured after bombardment into petals from various mutants, with or without the *Lc* and *C1* fusion genes. All lines used were *an4*⁻. Mutations affecting pigmentation and gene expression in the flower limb are indicated in the figure (Table 1). All bombardments were repeated at least 25 times within at least five independent experiments. In this figure, representative results are shown. See Table 2 for the averaged results.

genes control the expression of only a subset of biosynthetic genes (*chsJ*, *dfr*, *an13*, *rt*, and *amt*, but not *chsA*, *chi*, and *f3h*; Figures 2 and 3). This may be due to differences in the promoters of the homologous target genes from petunia and maize or the (functionally) homologous regulatory proteins. This led us to investigate whether *Lc* and *C1* differentially activate the promoter of the *chsA* (*an1*, *an2*, *an4*, and *an11* independent) and *chsJ* genes (*an1*, *an2*, *an4*, and *an11* dependent). In parallel experiments, we bombarded petunia leaves with *chsJ–gus*, *chsA–gus*, and, as a control, *dfrA–gus* fusion genes. As shown in Figure 8, we observed for all three reporter genes a small number of expressing cells, similar to the earlier experiments shown in Figure 6F. When the *dfrA–gus* or the *chsJ–gus* gene was cobombarded with the 35S–*Lc* and the 35S–*C1* gene, the number of blue spots increased, indicating that the activity of these reporter genes was induced by *Lc* and *C1*. However, such a large increase in activity was not observed for the *chsA–gus* fusion gene, indicating that *Lc* and *C1* cannot activate the *chsA* promoter.

This suggests that the Lc protein and its petunia homolog differentially activate *chs* promoters in the same way and that the choice of target genes, therefore, resides in the *chs* promoters.

DISCUSSION

an1, *an2*, *an4*, and *an11* Control Transcription of Structural Anthocyanin Biosynthetic Genes

In this study, we present a characterization of regulatory genes that control pigmentation of floral organs in petunia. We show that the structural anthocyanin genes, encoding enzymes of the pathway, are regulated in at least two separate units. Expression of *chsJ*, *dfr*, *an13*, *rt*, and *amt* in the petal limb is dependent on the action of the regulatory genes *an1*, *an2*, and *an11*, whereas expression of *chsA*, *chi*, and *f3h* is virtually independent of *an1*, *an2*, and *an11*. The anther-specific pigmentation gene *an4* also appears to be a regulatory gene because it controls the accumulation of *chsJ*, *dfr*, and *rt* mRNAs.

The *an1*, *an2*, and *an11* genes activate their target genes at the transcriptional level, because nascent *dfrA* and *rt* mRNA and transient expression of a *dfrA–gus* gene cannot be detected in corolla limbs of the corresponding mutants (Figures 3 and 6A to 6E). The reduced *chsJ* mRNA level in anthers of *an4*$^-$ plants is consistent with the low activity of a *chsJ–gus* transgene in anthers of *an2*$^-$/*an4*$^-$ plants (Koes et al., 1990), suggesting that *an4* also acts by transcriptional activation of structural genes.

We observed no effect of an *an10*$^-$ mutation on the expression of the structural genes (Figures 2 to 4, and 6D), indicating that *an10* is not a regulatory gene. This is in contrast to the data of Beld et al. (1989), who showed a reduced steady state level of *dfr* mRNA in corolla of an *an10*$^-$ line. To clarify this discrepancy, we have analyzed the flavonoids accumulating in *an10*$^-$ mutant corolla limbs and performed flavonoid precursor feeding experiments (data not shown). The absence of 4-coumaroyl coenzyme A derivatives, such as caffeic acid, in *an10*$^-$ flowers and the restoration of flower pigmentation after feeding with naringenin (Figure 1) indicate that *an10* controls one of the very early steps of the pathway. One way to explain the discrepancy is that Beld et al. (1989) may have unwittingly used a double mutant.

The division of the anthocyanin pathway in two separately regulated units is well conserved in snapdragon. However, the

Figure 6. (continued).

(A) Bombardment of *dfrA–gus* into petals from W80.
(B) Bombardment of *dfrA–gus* into petals from W162.
(C) Bombardment of *dfrA–gus* into petals from W115.
(D) Bombardment of *dfrA–gus* into petals from W154.
(E) Bombardment of *dfrA–gus* into petals from W134.
(F) Bombardment of *dfrA–gus* into a mature leaf of W80.
(G) Bombardment of *dfrA–gus* into a mature leaf of W80.
(H) Bombardment of *dfrA–gus*, *Lc*, and *C1* into a young leaf of W80.
(I) The region boxed in **(H)** is at a higher magnification.
(J) Bombardment of *dfrA–gus*, *Lc*, and *C1* into petals of W162.
(K) Bombardment of *dfrA–gus*, *Lc*, and *C1* into petals of W115.
(L) Bombardment of *dfrA–gus*, *Lc*, and *C1* into petals of W134.
(M) Bombardment of *dfrA–gus* and *Lc* into petals of W162.
(N) Bombardment of *dfrA–gus* and *Lc* into petals of W115.
(O) Bombardment of *dfrA–gus* and *Lc* into petals of W134.
(P) Bombardment of *dfrA–gus* and *C1* into petals of W162.
(Q) Bombardment of *dfrA–gus* and *C1* into petals of W115.
(R) Bombardment of *dfrA–gus* and *C1* into petals of W134.
(S) Bombardment of *dfrA–gus* and *C1* into petals of W80.
(T) Bombardment of *dfrA–gus* and *C1* into petals of W162.
In **(M)**, **(R)**, and **(T)**, arrows indicate spots appearing at low frequency.

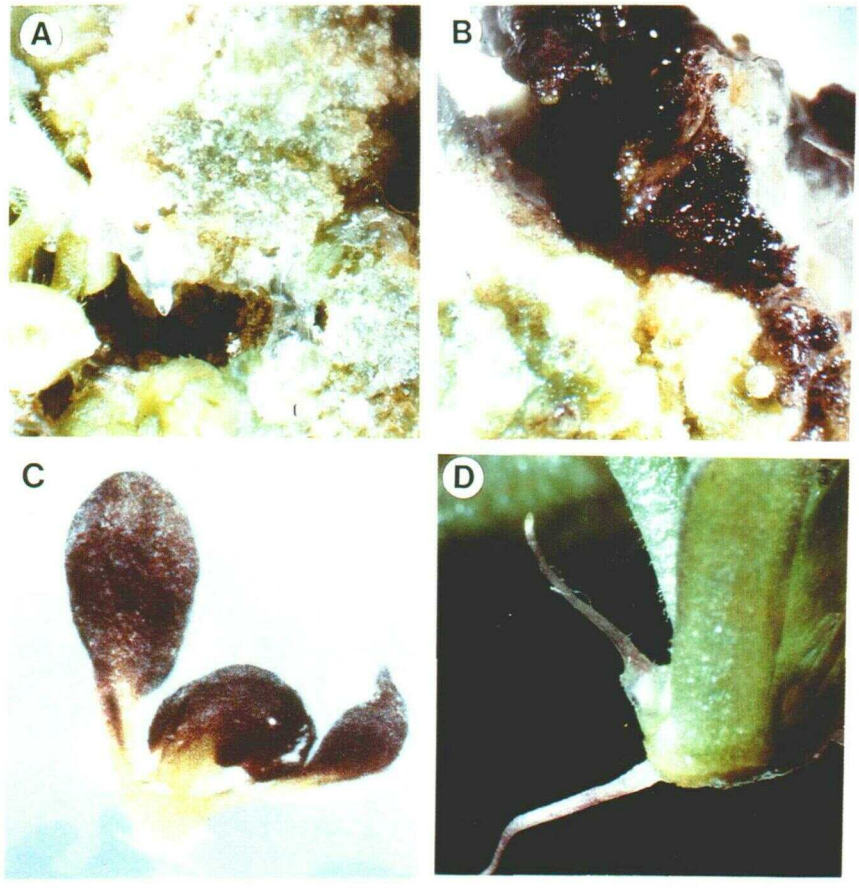

Figure 7. Agrobacterium-Mediated Transformation of Petunia V26 with an *Lc* Fusion Gene.

(A) Untransformed callus tissue from V26 leaf discs.
(B) Callus tissue from V26 leaf discs transformed with the *Lc* fusion gene.
(C) Pigmented shoot regenerated from a V26 leaf disc transformed with the *Lc* fusion gene.
(D) V26 plantlet transformed with the *Lc* fusion gene displaying pigmentation in the roots.

division occurs before *f3h* (and not thereafter, as in petunia) (Martin et al., 1991). In maize kernels, on the other hand, the structural genes are regulated as a single unit (Dooner et al., 1991). This point will be discussed below.

Genes That Regulate Pigmentation of Maize Kernels Induce Pigmentation in Petunia

To test whether regulatory pigmentation genes from maize are able to induce structural pigmentation genes from petunia, we introduced a 35S–*Lc* construct in petunia via Agrobacterium transformation. Although we were not able to regenerate healthy *Lc*-expressing plants, our data show that *Lc* can induce pigmentation in petunia in callus tissue and young plantlets (Figure 7). In the course of these experiments, Lloyd et al. (1992) reported that constitutive expression of an *Lc* transgene in Arabidopsis and tobacco plants results in intensified pigmentation, but does not alter the pattern of pigmentation. In most of their experiments, Lloyd et al. (1992) used a construct containing a full-size *Lc* cDNA, which includes the 5′ untranslated region. This region of the *R* genes has been recently reported to contain several short open reading frames plus a loop structure that causes a reduction in translation efficiency (Consonni et al., 1993; Damiani and Wessler, 1993). The construct we used lacked the 5′ untranslated region and, hence, is expected to direct synthesis of far larger quantities of Lc protein. This may explain the deleterious effects that we observed. Because the Lc protein contains an HLH motif, it can sequester endogenous HLH transcription factors by dimerizing with them, resulting in a complex with aberrant activity or none at all. We switched to transient expression assays

because these allowed us to assay the induction of a single (reporter) gene by *Lc* and *C1* in wild-type and mutant tissues.

Several observations indicate that the transient expression assays are specific and faithfully reflected the in vivo situation. First, *dfrA–gus* was transiently expressed after introduction in corollas from *an6*$^-$ and *an10*$^-$ petunia lines but not in those of *an1*$^-$, *an2*$^-$, and *an11*$^-$ lines, whereas a *chsA–gus* gene was active in all these lines. This is consistent with the transcription run-on and the RNA gel blot analyses. Furthermore, it is consistent with the data demonstrating that the expression of a stably introduced *dfrA–gus* transgene in flower corolla of petunia is strongly down-regulated after crossing it into an *an1*$^-$ and *an2*$^-$ genetic background (H.S.M. Huits, M.M. Kreike, J.N.M. Mol, A.G.M. Gerats, and R.E. Koes, unpublished data). Second, the combination of *Lc* and *C1* can activate the *dfrA* and the *chsJ* promoter in leaf cells, but not that of *chsA*, which is consistent with the different genetic control of these three genes. Third, *Lc* and *C1* can reactivate the *dfrA* promoter in corollas of specific petunia mutants only: *Lc* can (re)activate the *dfrA–gus* gene in an *an2*$^-$ background (Figure 6N; Table 2), whereas *C1* is not effective in this mutant (Figure 6Q). *C1*, however, can restore the activity of the *dfrA* promoter in the *an11*$^-$ mutant (Figure 6R).

Bowen (1992) showed that in particle bombardment experiments, the number of visually detectable spots is a reliable measure for the expression level of the reporter gene. Nevertheless, variation is observed among different shots, and, therefore, we only interpreted the number of spots as a rough measure (in terms of high or low expression; see Table 2). For a more precise quantitation, GUS activity should be measured in quantitative fluorescence assays and should be related to the expression of an internal standard (e.g., a cobombarded luciferase gene).

Table 2. Expression of the *dfrA–gus* Construct Transformed either Alone or in Combination with *C1* and/or *Lc* Constructs in Corolla Limbs of Different Petunia Mutants

		Expression of *dfrA–gus* after Codelivery with[b]			
Line	Mutation[a]	None	*Lc*	*C1*	*Lc+C1*
W80	an6$^-$	+ + +	+ + +	+ + +	+ + +
W162	an1$^-$	–	+/–	–	+ + +
W115	an2$^-$	–/+	+ +	–	+ + +
W154	an10$^-$	+ + +	+ + +	+ + +	+ + +
W134	an11$^-$	–	–	+	+ + +

[a] Only mutations relevant for pigmentation of the corolla are given.
[b] All bombardments were repeated at least 25 times within at least five independent experiments. The expression is given as the number of blue spots per bombarded corolla: + + +, wild-type level of expression (50 to 300 spots per shot); + +, high level of expression (20 to 50 spots per shot); +, low level of expression (5 to 10 spots per shot); +/–, a few spots appearing sporadically; –, no expression.

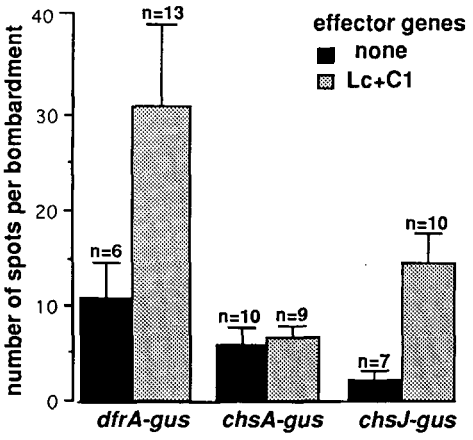

Figure 8. Induction of the *chsA*, *chsJ*, and *dfrA* Promoters by *Lc* and *C1* in Transient Expression Assays.

Shown are the number of blue spots observed when the *dfrA–gus*, *chsJ–gus*, or *chsA–gus* fusion genes were introduced by way of particle bombardment in petunia leaves with or without the 35S–*Lc* and 35S–*C1* constructs.

Role of *an1*, *an2*, *an4*, and *an11* in Transcription Activation of Pigmentation Genes

The efficient complementation of the *an2*$^-$ mutant with the *Lc* gene indicates that *an2* encodes or controls a transcription factor functionally homologous to *Lc*. We recently isolated from petunia (by way of polymerase chain reaction) an *Lc*-homologous cDNA fragment whose mRNA is down-regulated in *an2*$^-$ corolla limbs, but not in those from *an1*$^-$ and *an11*$^-$ mutants, supporting this conclusion (F. Quattrocchio, J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes, unpublished data). The observation that *Lc* can reactivate the *dfrA* promoter in *an4*$^-$ anthers (Figure 6T) suggests that *an4* and *an2* may be two differentially expressed members of a gene family, similar to the *R* family from maize (Chandler et al., 1989; Robbins et al., 1991; Tonelli et al., 1991). The gene *an11* is the most likely candidate to encode or control a C1-homologous protein, because an *an11*$^-$ mutant can be complemented by *C1* alone, albeit at low efficiency.

We observed that complementation of the *an2*$^-$ mutant by *Lc* is enhanced by codelivery of *C1* and that complementation of the *an11*$^-$ mutant by *C1* is enhanced by *Lc*. This can be explained in two ways. First, a functional analysis in yeast cells of the protein encoded by the maize *B* gene, which is homologous to *Lc* (Chandler et al., 1989), indicated that it can interact directly with the C1 protein (Goff et al., 1992). When *Lc* alone is expressed in *an2*$^-$ flowers, activation of the *dfrA* promoter depends on the interaction of the Lc protein with an endogenous (C1-homologous) transcription factor. The interaction of Lc with the petunia C1 homolog may be less efficient than with

C1 itself or the ratio between the Lc and C1 protein (or its petunia homolog) may be an important factor, as has been reported for transactivation of the *Bronze-2* (*Bz2*) promoter in maize protoplasts (Bodeau and Walbot, 1992). Second, accumulation of anthocyanins in the petunia flower is restricted to epidermal cells (Koes et al., 1990). Hence, expression of the regulatory genes is expected to be epidermis specific, as has been shown for the expression of *delila* in snapdragon flowers (Goodrich et al., 1992). If this is the case, reactivation of the *dfrA* promoter by *Lc* only occurs in cells expressing the C1-homologous gene (epidermal cells), whereas reactivation by *C1* plus *Lc* may occur both in epidermal and mesophyll cells. At present, we cannot differentiate between these two possibilities.

The nature of the *an1* gene product and its role in transcription regulation of the structural genes are unclear at present. The *an1* mutation is efficiently complemented by *Lc* plus *C1*, but not by *C1* alone and only at very low efficiency by *Lc*. One way to explain the complementation data is to assume that *an1* acts as a regulator of the expression of the petunia *Lc*- and *C1*-homologous genes. However, the finding that the mRNA of an *Lc*-homologous gene from petunia is down-regulated in *an2*$^-$ flowers but not in *an1*$^-$ flowers argues against such a role for *an1* (F. Quattrocchio, J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes, unpublished data). Additional experiments are required to establish the exact role of *an1*.

Transient expression experiments in leaves (Figure 6G) show that *Lc* plus *C1* is sufficient to induce the *dfrA* promoter in a tissue in which it is normally silent (e.g., Figure 4). This indicates that the petunia homologs are important factors in determining tissue-specific *dfrA* expression. Induction of pigment synthesis obviously requires the activation of all structural genes of the anthocyanin pathway. Although *an1*-, *an2*-, and *an11*-regulated genes, like *dfrA* and *chsJ*, are induced by *Lc* plus *C1*, separately regulated genes (like *chsA* and presumably *chi* and *f3h*) are not (see also Figure 8), which explains why no induction of anthocyanin pigmentation was observed. Because petunia leaves do contain dihydroflavonols and flavonols (Figure 1), the early genes of the pathway (*chs*, *chi*, and *f3h*) must be active at some stage in leaf development. This might explain why pigmented cells are observed only in very young leaves after bombardment with *Lc* plus *C1*. If in such cells *chs*, *chi*, and *f3h* genes are activated by endogenous factors, the activation of *dfrA* and other *an1*-, *an2*-, and *an11*-controlled genes by *Lc* and *C1* would be sufficient for anthocyanin production.

Evolution of the Flavonoid Pathway and Its Regulators: A Model That Explains Species-to-Species Similarities and Dissimilarities

Based on the sequence homology between the *delila* and *Lc* genes, Goodrich et al. (1992) argued that both are derived from a common ancestral gene and that subsequent alterations in their promoters are responsible for the different pigmentation patterns of maize and snapdragon. In this view, the functional *Lc* homolog from petunia may be derived from the same ancestor and similarly *C1* and its petunia homolog may have a common origin. If this is true, it raises the question of how these regulators have obtained such different sets of target genes.

Analyses of the distribution of flavonoids among plants species indicate that different sets of flavonoid biosynthetic genes appeared at distinct time points during evolution (Swain, 1986; Stafford, 1991). Chalcones and flavanones are the most ancient flavonoids and arose ~500 million years ago. The first flavonols appeared ~370 million years ago, whereas anthocyanins appeared later again (120 million years ago). This implies that *chs* genes were the first flavonoid genes, followed later by *f3h* (flavonol biosynthesis) and again later by anthocyanin biosynthetic genes, such as *dfr*, *an13*, *uf3gt*, *rt*, and *amt*. Because the isomerization of chalcones also occurs spontaneously, it is unclear at which time point *chi* genes arose (Stafford, 1991). It is thought that the structural flavonoid genes are derived from genes encoding enzymes in primary metabolism following gene duplication. For example, CHS and several fatty acid biosynthetic enzymes are thought to have derived from a common ancestral condensing enzyme (Kaupinen et al., 1988; Tsay et al., 1992; Verwoert et al., 1992), whereas *dfr* genes may share a common ancestor with dehydrogenases involved in steroid metabolism (Baker, 1991). Obviously, the gene duplication event must have been followed by mutations in the protein coding sequences and the regulatory sequences to optimize the encoded enzyme and to have the gene expressed at the right time and place. In the case of anthocyanin-specific genes (for example, *dfr*, *an13*, *uf3gt*, and *rt*), a crucial step must have been the coupling to the ancestors of today's *C1* and *Lc* (and their homologs).

Compatible modifications in the regulatory sequences must have followed, otherwise anthocyanin biosynthesis would be restricted to flavonol- or flavanone-synthesizing cells that already express *chs* and *f3h*. For the required adaptations in *chs* and *f3h* expression pattern, two mechanisms, or a combination of both, can be envisaged. First, novel *cis*-acting elements may have been added to the *chs* and *f3h* promoter, resulting in chimeric promoters that respond to distinct *trans*-acting factors in different tissues. Alternatively, *chs* and *f3h* genes may have been duplicated, followed by coupling of one set of gene copies to the regulatory *C1* and *Lc* / *delila* / *an2* ancestors. This results in two sets of early acting genes: one that is coordinately controlled with the anthocyanin-specific genes *dfr*, *uf3gt*, and *rt* (type A genes), and a second set that is still under the original control, possibly coordinated with flavonol- or flavanone-specific genes (type F). During later stages of evolution, the type A or type F copy of a certain gene may have been selectively inactivated or deleted, as shown in Figure 9.

The generation of novel patterns of tissue-specific expression by a combinatorial contribution of multiple *cis*-acting elements has been reported for the CaMV 35S promoter (Benfey and Chua, 1990; Benfey et al., 1990), and there is some

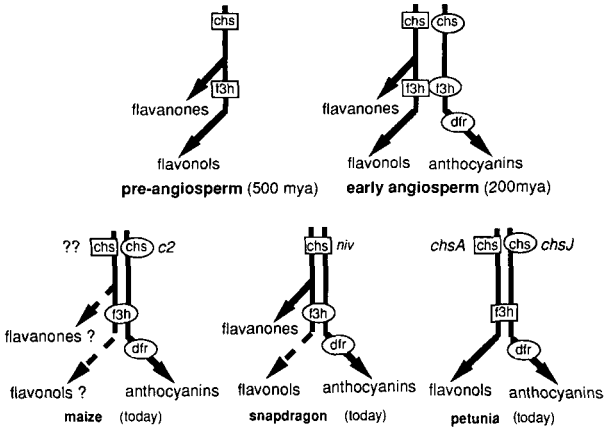

Figure 9. Model That Describes How Regulatory Genes in Petunia, Snapdragon, and Maize with a Common Evolutionary Origin May Have Acquired Different Sets of Target Genes.

Flavonoid genes have been classified as type A (gene names in ellipses) based on the genes that control their expression: *C1* and members of the *R* family from maize (Dooner et al., 1991), *delila* from snapdragon (Martin et al., 1991), or *an1*, *an2*, and *an11* from petunia (this study). Genes expressed independently from these regulatory genes have been classified as type F (gene names in squares). *niv*, *niv*ea locus; *C2*, *C2* locus; mya, million years ago.

evidence for a modular organization of flavonoid promoters (Leyva et al., 1992; van der Meer et al., 1992a, 1992b). Several findings suggest that at least in some cases, gene duplications have been involved; the *chsJ* (type A) and *chsA* (type F) genes from petunia are the clearest examples. Maize contains at least two *chs* genes, located at the *C2* and *White pollen* (*Whp*) loci (Franken et al., 1991). The *chs* gene at the *C2* locus is responsible for the pigmentation of most plant tissues and is controlled by *C1* and *R* genes (Dooner, 1983; Ludwig et al., 1989; Tonelli et al., 1991), making it a clear representative of type A genes. In maize seedlings, however, a *chs* transcript accumulates independently of *R* genes, suggesting the presence of a type F *chs* gene, although the chromosomal locus is not identified (Taylor and Briggs, 1990). Both petunia and snapdragon have a single *f3h* gene (Britsch et al., 1991; Martin et al., 1991). The *f3h* gene from snapdragon is controlled by *delila* (type A) (Martin et al., 1991), whereas the petunia *f3h* gene is expressed independently of *an2* (type F). This difference in regulation may be explained by the selective loss of the type A or type F copy of *f3h* in petunia and snapdragon, respectively. This may be connected to the observation that flavonols, which require F3H for their biosynthesis, are end products of flavonoid biosynthesis in petunia, whereas in snapdragon, flavanones (which do not require F3H for their synthesis) rather than flavonols are accumulated (Bartlett, 1989).

The above model predicts that the different control of *chs* and *f3h* gene expression in petunia, maize, and snapdragon is caused by differences in *cis*-acting elements within the *f3h* and *chs* genes and not by differences between *R* genes, *delila*, and the petunia homolog. This is consistent with the observation that *Lc* and *C1* cannot activate the *chsA* gene from petunia (Figure 8). The availability of cloned *Lc* homologs from snapdragon (Goodrich et al., 1992) and petunia (F. Quattrocchio, J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes, unpublished data) makes it possible to test the above model by swapping homologous regulatory genes between different species. Such analyses may provide us with novel insights into the development and evolution of regulatory networks.

METHODS

Plant Materials

The petunia lines that have been used are described in Table 1. All petunia lines were maintained by inbreeding, except for VR, which is an F_1 hybrid between lines V23 and R51. Lines R27, W138, W137, and W134 are fully isogenic because their different genotypes are the result of transposon insertions and excisions at the *Anthocyanin-1* (*An1*) and *An11* loci (Doodeman et al., 1984a, 1984b). A sporogenic revertant plant isolated from the *an2*-mutable line W82 was used as a (isogenic) wild type. All plants were grown under standard greenhouse conditions.

RNA Analyses

Corollas and anthers were dissected from flowers of a defined developmental stage, frozen in liquid nitrogen, and stored at −70°C until further use. Total RNA was extracted as previously described (van Tunen et al., 1988). For RNA gel blot analysis, 10 μg of RNA was loaded on formaldehyde agarose gels (van Tunen et al., 1988). After electrophoresis, RNA gels were stained for 2 min in 50 mM NaOH, 10 mM NaCl, 0.5 mg/L ethidium bromide, destained in 10 mM Tris-HCl, pH 7.5, and blotted onto Hybond-N membranes (Amersham). cDNA fragments encoding the following flavonoid biosynthetic enzymes were used as a probe: chalcone synthase (CHS) (Koes et al., 1989b), chalcone flavanone isomerase (CHI) (van Tunen et al., 1988), flavanone 3-hydroxylase (F3H) (kindly provided by L. Britsch, University of Freiburg, Germany and G. Forkmann, University of Munich, Germany) (Britsch et al., 1991), dihydroflavonol 4-reductase (DFR) (Beld et al., 1989), anthocyanin rhamnosyltransferase (RT) (Kroon et al., 1993), anthocyanin methyltransferase (AMT) (E. Souer, J.T.M. Kroon, B. Oppedijk, J.N.M. Mol, and R.E. Koes, unpublished data), and AN13, a yet undefined enzyme encoded by the *An13* locus (E. Souer, J.T.M. Kroon, B. Oppedijk, J.N.M. Mol, and R.E. Koes, unpublished data). A partial cDNA clone encoding phenylalanine ammonia-lyase was picked up as a false positive in a screen of a V26 corolla cDNA library for helix-loop-helix protein encoding cDNAs. The sequence of the fragment displayed an homology of 85% at the protein level with the *pal* sequence from bean (F. Quattrocchio, J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes, unpublished data).

RNase protection assays were performed according to the method of Koes et al. (1989a), except that the *chsA*-specific probe covered only the most 3′ 980 nucleotides of the mRNA sequence. To check for specificity of the assay, both *chsA* and *chsJ* probes were hybridized

to single-stranded DNA obtained from *chsA* and *chsJ* subclones in M13 vectors (data not shown).

Transcription run-on assays were performed as described previously (Weiss et al., 1992). In control experiments, α-amanitin (Sigma) was added to the nuclei at a final concentration of 4 mg/L prior to elongation of nascent transcripts.

Description of Fusion Genes

The *dfrA*–β-glucuronidase (*gus*) fusion gene was constructed as a translational fusion of a 2067-bp *dfrA* genomic fragment (1949 bp of promoter, with 82 bp of 5′ untranslated region and 36 bp of protein coding region) with the *gus* gene from pBI221 and the nopaline synthase (*nos*) tail of the same plasmid. The *chsA–gus* gene construct (Vip161) has been described before (Koes et al., 1990). The cauliflower mosaic virus 35S–*Leaf color–nos (CaMV* 35S–*Lc–nos*) construct, pSRL349 (kindly provided by S. Wessler, University of Georgia, Athens) contains the *Lc* cDNA fused to the CaMV 35S promoter and the *nos* tail fragment of pBI221 (Ludwig et al., 1989). The C1 protein coding region was isolated from the plasmid p35SC1 (kindly provided by S. Goff and V. Chandler, University of Oregon, Eugene) (Goff et al., 1990) by digestion with BamHI and SstI and ligated in PBI221 (Jefferson et al., 1987), from which the *gus* coding sequence had been removed by digestion with the same enzymes. The resulting plasmid, Zip701, contains the C1 protein coding region fused to the CaMV 35S promoter and the *nos* 3′ tail.

Transient Expression Assays Using Particle Bombardment

Flower buds at stages 3 to 4 (Koes et al., 1989a) and leaves were collected, rinsed in 70% ethanol for a few seconds, and sterilized by immersion for 10 min in 3% sodium hypochlorite. After several washings in sterile water, the sepals were removed and the corollas were cut transversely, unrolled, and placed on solidified (0.7% agar) Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 3% sucrose. After a 24-hr incubation at 24°C, tissues were bombarded with DNA-coated tungsten particles using the PDS-1000/He particle gun (Du Pont).

Tungsten microprojectiles (1.8 μm; kindly provided by Pioneer Hi-Bred International Inc., Johnston, IA) were resuspended in 50 μL of distilled water. Five micrograms of plasmid DNA, 50 μL $CaCl_2$ (2.5 M), and 20 μL spermidine-free base (0.1 M) were added to the particles and mixed by vortexing for ~5 min. The particles were subsequently centrifuged and the supernatant was removed. After washing once in 100% ethanol, the particles were resuspended in 60 μL of 100% ethanol. For every bombardment, 10 μL of coated particles were spotted on the macroprojectile and allowed to dry in a flow cabinet. Bombardments were performed using a pressure of 1550 psi. After transformation, the tissues were incubated for 48 hr at 25°C in a growth cabinet and stained for GUS activity as described previously (Jefferson et al., 1987). After 48 hr of staining at room temperature and overnight destaining in 70% ethanol, spots were counted using a dissection microscope.

Corollas and leaves from each petunia line utilized in the assay were transformed with a *chsA–gus* gene to verify that the transformation efficiency of the different mutants was at least comparable (data not shown).

Permanent Transformation

For permanent transformation, constructs were recloned into the binary vector Bin19 (Bevan, 1984), and transgenic plants were obtained via leaf disc infection with *Agrobacterium tumefaciens* as previously reported (Quattrocchio et al., 1990).

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