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Chalcone Synthase Promoters in Petunia Are Active in Pigmented and Unpigmented Cell Types

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Chalcone synthase (CHS) catalyzes the first step in the biosynthesis of flavonoids that function in flower pigmentation, protection against stress, and induction of nodulation. The petunia genome contains eight complete *chs* genes, of which four are differentially expressed in floral tissues and UV-light-induced seedlings. The 5′-flanking regions of these four *chs* genes were fused to the β-glucuronidase (GUS) reporter gene and introduced into petunia plants by *Agrobacterium*-mediated transformation. We show that expression of each construct is identical to the expression of the authentic *chs* gene, implying that the differences in expression pattern between these *chs* genes are caused at least in part by their promoters. Histochemical analyses of GUS expression show that *chs* promoters are not only active in pigmented cell types (epidermal cells of the flower corolla and tube and [sub] epidermal cells of the flower stem) but also in a number of unpigmented cell types (mesophylic cells of the corolla, several cell types in the ovary and the seed coat). Comparison of *chs*-GUS expression and flavonoid accumulation patterns in anthers suggests that intercellular transport of flavonoids and enzymes occurs in this organ. Analysis of the flavonoids accumulated in tissues from mutant lines shows that only a subset of the genes that control flavonoid biosynthesis in the flower operates in the ovary and seed. This implies that (genetic) control of flavonoid biosynthesis is highly tissue specific.

INTRODUCTION

One of the most studied biochemical pathways in plants is the biosynthesis of flavonoids. Flavonoids are phenylpropanoid-derived secondary metabolites that have a key function in the pigmentation of flowers and fruits (anthocyanins and flavonols; Brouillard, 1988), the defense against phytopathogens (isoflavonoids; Dixon, 1986; Lamb et al., 1989), the protection against UV light (flavonols; Schmelzer et al., 1988), and the induction of nodulation (flavones; Long, 1989). Furthermore, they have been implicated in the regulation of auxin transport (flavonols; Jacobs and Rubery, 1988) and resistance to insects (flavanones, anthocyanins; Hedin and Waage, 1986), and it is likely that even more functions of flavonoids remain to be discovered.

Flavonoids are synthesized by way of a side branch of the general phenylpropanoid pathway, naringenin-chalcone being the first specific intermediate. The naringenin chalcone is formed by means of condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA catalyzed by the enzyme chalcone synthase (CHS). Subsequent isomerization of naringenin-chalcone by chalcone-flavanone isomerase (CHI) yields naringenin-flavanone, the central intermediate of the flavonoid pathway. From this point the pathway diverges into several side branches, each one yielding a different class of flavonoids (see Heller and Forkmann, 1988; van Tunen and Mol, 1989, for reviews).

Because color is a convenient phenotypic marker, the biochemistry and genetics of genes involved in the synthesis of flavonoid pigments (anthocyanins) have been studied extensively. More recently, a large number of these genes have been cloned and characterized at the DNA level (see Mol et al., 1988, for a listing). The multitude of functions of flavonoids requires a correspondingly complex regulation of genes encoding different enzymes of the pathway. In normal healthy plants, expression of genes of anthocyanin biosynthesis is flower specific, light dependent, and developmentally controlled (van Tunen et al., 1988; Koes et al., 1989a). Expression in other tissues can be induced, however, by UV light, wounding, or fungal attack (Dixon, 1986; Koes et al., 1989a; Lamb et al., 1989). Several genes from the pathway are regulated at the transcriptional level in response to these different stimuli (Chappell and Hahlbrock, 1984; Cramer et al., 1984; Lawton and Lamb, 1987). The tissue specificity and inducibility in combination with the well-developed genetics and biochemistry make flavonoid biosynthetic genes an ideal model system to study regulation of gene expression at the molecular level. Recent research has begun to identify *cis*-acting elements and *trans*-acting factors involved in the regulation of fla-

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vonoid biosynthetic genes (Schulze-Lefert et al., 1989; Staiger et al., 1989).

Detailed knowledge of the genetics and biochemistry of the flavonoid pathway and the availability of cloned genes recently led to the development of molecular flower breeding (see Mol et al., 1989a, 1989b, for overviews). Introduction of a dihydroflavonol 4-reductase gene (encoding the first specific enzyme of the anthocyanin pathway) from maize in transgenic petunia resulted in flowers with a novel, brick red color due to the synthesis of pelargonidins, a type of anthocyanin that normally is not synthesized in petunia (Meyer et al., 1987). Introduction of an antisense *chs* gene in transgenic petunia resulted in a block of pigmentation (van der Krol et al., 1988). In addition to plants with completely white flowers, plants that exhibited novel patterns (rings, sectors, etc.) of pigmentation (van der Krol et al., 1988) were also obtained.

Although a wealth of knowledge exists about the biochemistry, genetics, and molecular biology of the flavonoid pathway, relatively little is known about the expression of the genes at the cellular level. We believe that such information may lead to a better understanding of various aspects of flavonoid biosynthesis such as biological functions of flavonoids, the nature of *trans*-acting factors in a particular tissue (inducers, repressors, or a mixture of both), or mechanisms of pattern formation in (manipulated) plants. Therefore, we decided to study the cell-type-specific expression of genes encoding the two key enzymes of the pathway in petunia: chalcone synthase (CHS; this paper) and chalcone flavanone isomerase (CHI; van Tunen et al., 1990).

In the inbred petunia variety V30, *chs* genes comprise a small multigene family consisting of eight complete genes and several gene fragments (Koes et al., 1989b), which are localized on two different chromosomes (Koes et al., 1987). Only two of these *chs* genes (*chs*A and *chs*J) are active in floral tissues and during UV stress conditions in seedlings. Expression of *chs*A and *chs*J in floral tissues is coordinate, light dependent, and developmentally regulated (Koes et al., 1989a). Two other *chs* genes (*chs*B and *chs*G) are active in UV-stressed seedlings only, albeit at a low level. In this paper we report a detailed analysis of the expression of chimeric genes consisting of the β-glucuronidase (GUS) reporter gene and the 5′-flanking region of these *chs* genes at the macroscopic and microscopic levels.

RESULTS

Construction of Transgenic Petunia Plants Containing *chs*-GUS Fusion Genes

Using gene-specific RNase protection assays, we have shown previously that in *Petunia hybrida* V30, four members of the *chs* gene family are active genes (Koes et al., 1989a). The *chs*A and *chs*J gene are co-expressed in various floral tissues and in UV light-induced seedlings; *chs*B and *chs*G are silent in floral tissues but their expression is UV inducible in young seedlings. To study their expression in more depth, we fused the 5′-flanking region of each of these *chs* genes to the GUS reporter gene (Jefferson et al., 1987). The structure of the resulting fusion genes is depicted in Figure 1. In VIP161, VIP165, and VIP162 the ATG translation start of *chs*A, *chs*J, and *chs*G, respectively, was fused in frame to the ATG of GUS, resulting in the addition to the GUS protein of 12, 8, and 7 amino acids, respectively.

The *chs*B promoter fragment was fused in two different reading frames to GUS. Sequence comparison of the proteins specified by different *chs* gene family members of petunia V30 (Koes et al., 1989a) and cDNAs from several different species (Niesbach-Klösgen et al., 1987) shows that the amino terminus of the protein is conserved in length and sequence. *chs*B is exceptional in this respect because the ATG start codon is located 9 nucleotides upstream of its normal position and the first 12 codons are completely divergent from any other *chs* gene (Koes et al.,

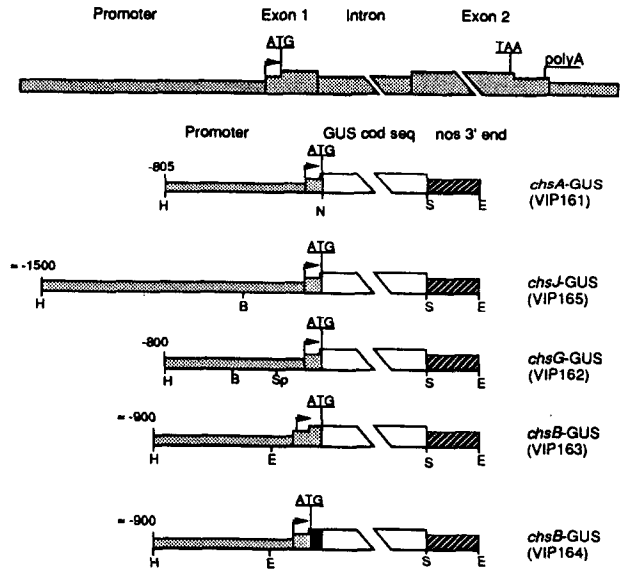

Figure 1. Generalized Organization of *chs* Genes in Petunia and Description of *chs*A-GUS, *chs*J-GUS, *chs*B-GUS, and *chs*J-GUS.

The upper drawing represents the generalized structure of *chs* genes from petunia V30 (Koes et al., 1989b). The structure of the *chs*-GUS fusion genes is given beneath. To indicate that the amino acids fused to GUS in VIP164 are not related to CHS, the corresponding DNA region is shown in black. As a landmark, some restriction sites and the length of promoters in nucleotides are given. Further details can be found in Methods. B, BamHI; E, EcoRI; H, HindIII; N, NcoI, S, SstI; Sp, SphI.

1989b). In VIP 163 this ATG is fused in frame with the ATG of GUS. A second ATG codon, which occurs 13 nucleotides downstream and out of frame to the first ATG, has high similarity to the consensus initiation codon. This second ATG specifies an open reading frame (without homology of the predicted protein to CHS proteins) that can, after 56 codons, be spliced theoretically to the second exon of the same gene and, hence, is potentially an active initiation codon. In VIP164 this second ATG is fused in frame to the ATG of GUS.

The different *chs*-GUS fusions were used to transform leaf discs of petunia. Because the petunia variety V30, from which the *chs* promoters were isolated, is recalcitrant to transformation and regeneration, we used petunia variety Mitchell as a host. The absence of anthocyanins in flowers of the white-flowering petunia variety Mitchell is caused by a block late in the anthocyanin pathway (conversion of dihydroflavonols to anthocyanidins) due to a mutation at the *An2* locus and does not interfere with expression of *chs* genes (Beld et al., 1989; see also Figure 6). This was confirmed by the observation that expression of *chs*A-GUS in the purple-flowering VR hybrid is identical to its expression in Mitchell at all levels (data not shown).

To determine the number of inserted copies of the constructs, DNA was extracted from leaves of individual transformants and analyzed by DNA gel blot hybridization using the GUS coding sequence as a probe. Based on the number of hybridizing border fragments, we estimated that the number of inserts varies from 1 to 8 in different transformants (data not shown).

Expression of *chs*-GUS Fusion Genes Is Coordinated with That of the Authentic *chs* Genes

To analyze the expression of the *chs*A-GUS fusion, we measured GUS activities in extracts prepared from various tissues of individual plants transformed with *chs*A-GUS(VIP161). For some transformants, we measured GUS activity in the flower corolla and anthers at different stages of flower development. A typical example of such an analysis is shown in Figure 2. In each of the transformants analyzed, GUS activity was low in anthers and the corolla of stage 1 flowerbuds. A strong increase of GUS activity occurred in anthers at stage 2 and later during development (stage 3/4) in the corolla and the tube. This matches well with RNA gel blot and RNase protection data showing that *chs*A mRNA levels peak at stage 2 in anthers and at stage 4 in the corolla and flower tube (Koes et al., 1989b). This implies that expression of *chs*A-GUS is developmentally controlled as the authentic *chs*A gene. The organ specificity of *chs*A is also maintained in *chs*A-GUS because none of the transformants tested showed GUS activity in leaves or stems (data not shown). The part of the stem that is immediately below the flower (flower stem) contained GUS activity, however, which is not surprising because this part of the stem is pigmented. This is also shown in Table 1.

To compare expression levels of *chs*A-GUS among different transformants, we measured GUS activities in stage 6 flowers because this developmental stage is morphologically well defined and GUS levels are maximal in anthers, corolla, and tube. Figure 3 shows that the expression levels of *chs*A-GUS vary considerably among transformants harboring the same constructs, and no correlation is found with the number of introduced copies. Such variations in expression level of a transgene are generally observed and are thought to be caused by influences of the bordering sequences, the so-called position effect (Weising et al., 1988). Analysis of the *chs*A-GUS expression in corolla by primer extension showed that the cap site of *chs*A-GUS mRNA maps at the same position as the authentic *chs*A mRNA, but that the mRNA levels are only about 1% of the endogenous *chs*A mRNA level in the highest expressors (data not shown).

Systematic histological analyses (see below) showed that the *chs*A-GUS gene was also expressed in unpigmented tissues such as ovaries and seedpods, which had

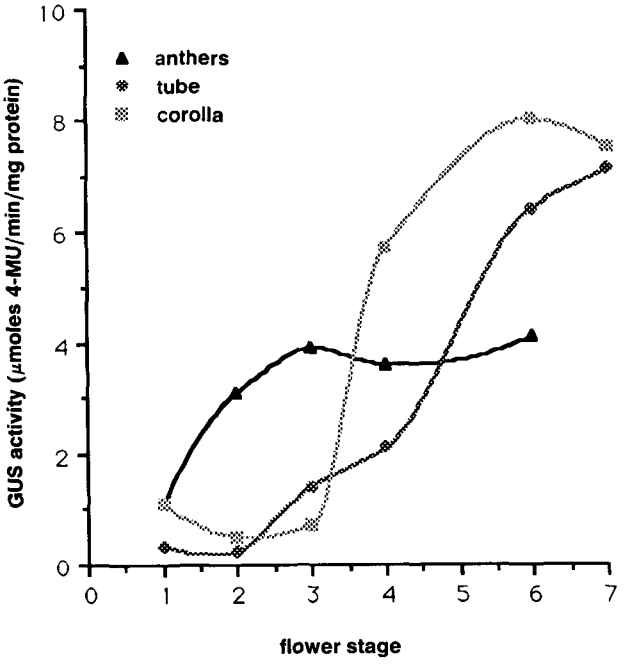

Figure 2. Expression of *chs*A-GUS in Flower Organs of Different Developmental Stages.

Flowers from different developmental stages were dissected in corolla, tube, and anthers and assayed for GUS activity. The division in developmental stages is based on morphological criteria as described by Koes et al. (1989a) for the V30 variety. 4-MU, 4-methylumbelliferyl.

Table 1. Types of Flavonols and Anthocyanins in Plant Tissues from Different Petunia Varieties

Variety	Recessive for	Tissue	Flavonol	Anthocyanin
VR	*mf2*	Corolla	Quercetin	Malvidin
		Flower stem	Quercetin	Malvidin
		Seed	+[a]	Delphinidin
V30	*fl, mf1, mf2*	Corolla	Quercetin[b]	Petunidin
		Ovary	Quercetin[b]	–[c]
		Seed	+	Delphinidin
R27	*hf1, hf2, rt*	Corolla	Quercetin	Cyanidin
		Ovary	Quercetin	–
		Seed	+	Delphinidin
W85	*an6, ht1, hf1, rt*	Corolla	Kaemferol	–
		Tube	Quercetin	–
		Ovary	Quercetin	–
		Seed	+	–
W80	*an6, ht1, ht2, hf1, hf2*	Corolla	Kaemferol	–
		Ovary	Kaemferol	–
		Seed	+	–
W78	*an1, hf1, hf2*	Corolla	Kaemferol	–
		Tube	Quercetin	–
		Ovary	Quercetin	–
		Seed	+	–
Mitchell	*an2, mf1, mf2*	Corolla	Quercetin	–
		Ovary	Quercetin	–
		Seed	+	Delphinidin

[a] +, flavonols detectable at very low levels insufficient to discriminate between kaemferol and quercetin.
[b] Present in low amount because of the recessive status of *fl*.
[c] –, not detectable.

not been analyzed previously by other methods. For several transformants we determined the amounts of GUS activity in these tissues quantitatively by fluorimetric assays. Figure 3 shows that specific GUS activities in ovaries and seedpods are at least as high as those in the corolla or tube of the flower. However, one cannot conclude directly that the expression per cell is much higher because neither the average amount of protein per cell nor the actual number of expressing cells in these tissues is known.

Expression of the *chs*J-GUS fusion exhibited the same organ specificity as the *chs*A-GUS fusion (Figure 3). It was expressed in the flower tube, corolla, ovaries, flower stem, and seedpods but not in leaves or stems. In anthers expression of *chs*J-GUS above the background was detected, although the expression levels were relatively low. Of the eight transformants containing *chs*B-GUS, only one showed a low level of expression in corolla, tube, anthers, flower stem, and ovary. This suggests that the *chs*B promoter is active at a very low level in these tissues. No activity of the *chs*G-GUS fusion could be detected by fluorimetric assay in tissues from several individual transformants, as expected. (DNA gel blot analysis did show that they contained the constructs and were not escapees from the kanamycin selection procedure.) This indicates that the *chs*G promoter was inactive in the tissues tested.

In summary, the analyses on the expression of the *chs*-GUS fusions at the macroscopic level show that they retained the regulation of the authentic *chs* gene. This implies that the differences in expression of these *chs* genes are at least in part caused by differences in their promoters and/or the leaders of their mRNAs.

Expression of *chs*-GUS Fusions in Structural Flower Organs

To localize the site of anthocyanin accumulation in flowers from petunia, we prepared cross-sections in which these pigments were still present. Figure 4A shows that the purple anthocyanins are accumulated in the inner epidermis of the flower tube and in the connective tissue plus surrounding cells. After incubation of cross-sectioned flower tubes (stage 4) of *chs*A-GUS transformants in X-gluc, a strong blue color, representing GUS activity, developed in the inner side of the tube (Figure 4B). Examination of the same tissue at the single-cell level showed that this GUS activity was localized mainly in cells of the inner epidermis and in the neighboring cells of the mesenchyme (Figure 4E). In addition, some GUS activity was observed in cells surrounding the veins. A similar pattern of staining was observed in plants transformed with *chs*J-GUS (Figure 4C). No blue staining was observed when flower tubes of *chs*G-GUS transformants or untransformed control plants were examined. This rules out that the blue staining is the result of endogenous GUS activity (Figure 4D). A similar analysis of flower tubes of a cauliflower mosaic virus (CaMV)-GUS-transformed plant showed that every cell type was stained and hence had access to the X-gluc substrate (Figure 4F). This implies that the staining pattern observed in *chs*A-GUS- and *chs*J-GUS-transformed plants represents the expression pattern of these genes and matches well with the pattern of anthocyanin accumulation.

Figure 4G shows that in the corolla, anthocyanins accumulated mainly in the inner epidermis, to a somewhat lesser extent in the outer epidermis, and were missing in the parenchymous cells in between. The expression patterns of *chs*A-GUS and *chs*J-GUS in corolla differed fundamentally from this anthocyanin accumulation pattern. Figure 4H shows that stage-4 corollas of *chs*A-GUS-transformed plants exhibited GUS activity in the outer and inner epidermis as well as in the parenchymous cells in between. The same expression pattern was observed at earlier stages of development (Figure 4I). Identical results were obtained for *chs*J-GUS-transformed plants (not shown).

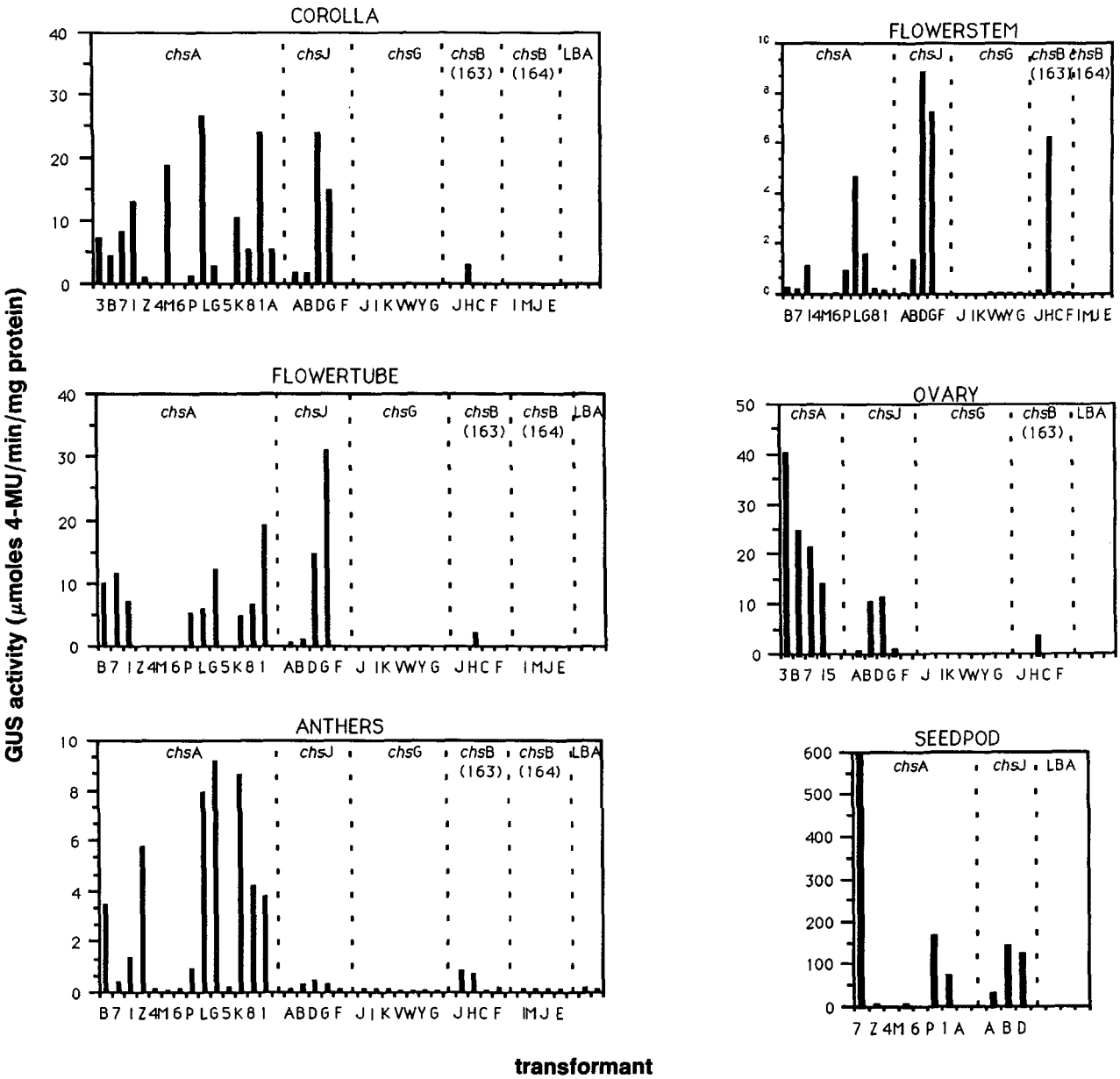

Figure 3. Expression of *chs*-GUS Fusions in Different Plant Tissues of Multiple Independent Transformants.

GUS activity was measured in corolla, tube, anthers, and ovaries of stage-6 flowers and the pigmented part of the stem immediately below the flower (flower stem) from plants independently transformed with VIP161 (*chs*A), VIP165 (*chs*J), VIP162 (*chs*G), VIP163 (*chs*B), VIP164 (*chs*B), or an *Agrobacterium* strain without a binary vector (LBA). GUS activity in seedpods was measured 10 days after pollination of each transformant with pollen from untransformed petunia Mitchell plants.

No staining was observed in *chs*G-GUS transformants or in untransformed plants, implying that no background GUS activity was present in this tissue.

In flower stems of *chs*A-GUS-transformed plants, GUS activity was localized in the outer five to six cell layers, including the epidermis (Figure 4J). Identical results were obtained with flower stems of *chs*J-GUS-transformed plants. No staining was observed in *chs*G-transformed plants or untransformed plants, showing that no background GUS activity was present in this tissue. The ob-

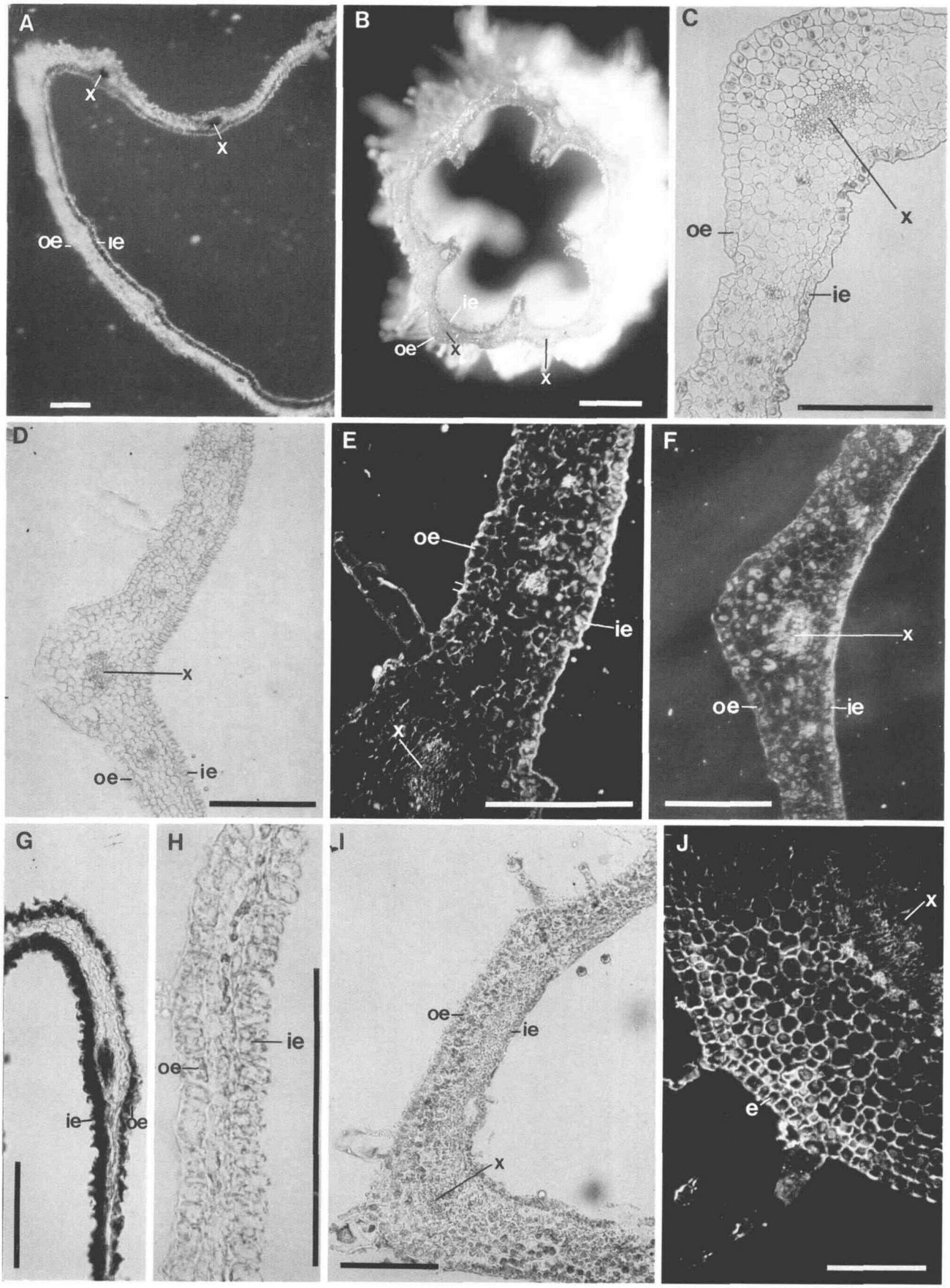

Figure 4. Histochemical Localization of Anthocyanins and GUS Activity in Flower Organs of Transformed Plants.

served expression patterns for *chs*A-GUS and *chs*J-GUS coincided with the site of anthocyanin accumulation in this tissue (not shown).

Expression of *chs*-GUS Fusions in Reproductive Organs

In cross-sections, 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 exotecium. 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. Depending on the genotype, anthers of most petunia varieties are pigmented. Figure 5A1 shows that in early stages of flower development (stage 1) the purple anthocyanins were localized mainly in the parenchymous cells of the connectivum. At later developmental stages (stage 4/5), the anthocyanins appeared to be more evenly distributed throughout the anther (Figure 5A2).

In anthers from stage-2 flowers of *chs*A-GUS-transformed plants incubated with X-gluc, intensive staining was observed in the outer parenchymatic cells of the connectivum, the tapetal cell layer, and in the pollen grains, but never in the connective tissue or the outer cells of the exotecium (Figures 5B1 and 5B2). Staining of pollen grains was also observed in stage 2 anthers from control and *chs*G-GUS-transformed plants, indicating that this is due to a background GUS activity (see Plegt and Bino, 1989, for more details). At later stages of anther development, this background GUS activity was considerably reduced, but still no staining above the background could be seen in pollen from *chs*A-GUS-transformed plants. This indicates that if *chs*A-GUS is expressed at all in pollen grains, it is not at high levels. At late developmental stages (stage 6), anthers of *chs*A-GUS transformants still contained high levels of GUS activity (Figures 2 and 3), but this GUS activity was not detectable in histological assays (Figure 5B6). In stage 4 anthers, small patches of blue staining were found in the remains of the degenerated tapetum and parenchymatic cells (Figure 5B5). This suggests that the GUS activity in these cells was not degraded after their degeneration, but was probably liberated into the locule.

Anthers from plants transformed with CaMV-GUS showed staining in every cell type upon incubation in X-gluc, indicating that all cells have access to the X-gluc substrate (Figures 5C1 and 5C2), but expression in the parenchymatic cells of the connectivum appeared to be lower than in other cell types (see Discussion).

In longitudinal sections of flowers from *chs*A-GUS and *chs*J-GUS transformants, we observed a very rapid and intense staining in the ovaries. Analysis of these tissues at the cell level showed that this GUS activity was localized in the ovules and to a lesser extent in the tissues immediately surrounding them (Figures 5E1 and 5E2). Using higher expressors and longer staining periods, blue staining was also observed in the capsule of the ovary (not shown), indicating that these cells also contained GUS activity, but at a lower level. No staining was observed in control plants or *chs*G-GUS-transformed plants even after prolonged staining, indicating that no background GUS activity was present in these cell types.

After pollination, high expression of the *chs*A-GUS and *chs*J-GUS constructs was detected in seedpods and, more specifically, in the seeds (Figure 5D1). At higher magnification, blue staining appeared to be localized in the hexagonally shaped cells of the seed coat (Figure 5D2). No GUS activity was detectable in the placenta and the capsule of the seedpod. When seeds were cross-sectioned, GUS activity was predominantly found in the cells of the seed coat (Figure 5D3). Overstaining of seeds from very high expressors (e.g., 161-7 see Figure 3) showed that the endosperm and the embryo also contained GUS activity, although at far lower levels (data not shown). Control seedpods from a transformant harboring a GUS gene driven by a maize seed storage protein (zein) promoter stained readily in the endosperm, indicating that this tissue had good access to the X-gluc substrate (Quattrocchio et al., 1990). No staining was observed in seedpods of untransformed or *chs*G-GUS-transformed plants (data not shown).

Figure 4. (continued).

(A) Localization of anthocyanins in a section of the flower tube of the variety V30.
(B) Macroscopic image of a transversely cut flower tube from a *chs*A-GUS transformant after staining with X-gluc.
(C) Bright-field micrograph of an X-gluc-stained flower tube from a *chs*J-GUS transformant.
(D) Bright-field micrograph of an X-gluc-stained flower tube from a plant transformed with an *Agrobacterium* strain without a binary vector.
(E) Dark-field micrograph of an X-gluc-stained flower tube from a *chs*A-GUS transformant.
(F) Dark-field micrograph of an X-gluc-stained flower tube from a CaMV-GUS-transformed plant.
(G) Localization of anthocyanins in the flower corolla of the V30 variety.
(H) Bright-field micrograph of an X-gluc-stained flower corolla (stage 4) from a *chs*A-GUS transformant.
(I) Bright-field micrograph of an X-gluc-stained flower corolla (stage 1/2) from a *chs*A-GUS transformant.
(J) Dark-field micrograph of an X-gluc-stained flower stem of a *chs*A-GUS transformant.
GUS activity is represented in bright-field micrographs by a blue stain and in dark-field micrographs by a red/pink stain. Scale bars equal 1 mm in **(B)** and 300 μm in all other panels. Abbreviations: e, epidermis; ie, inner epidermis; oe, outer epidermis; x, xylem.

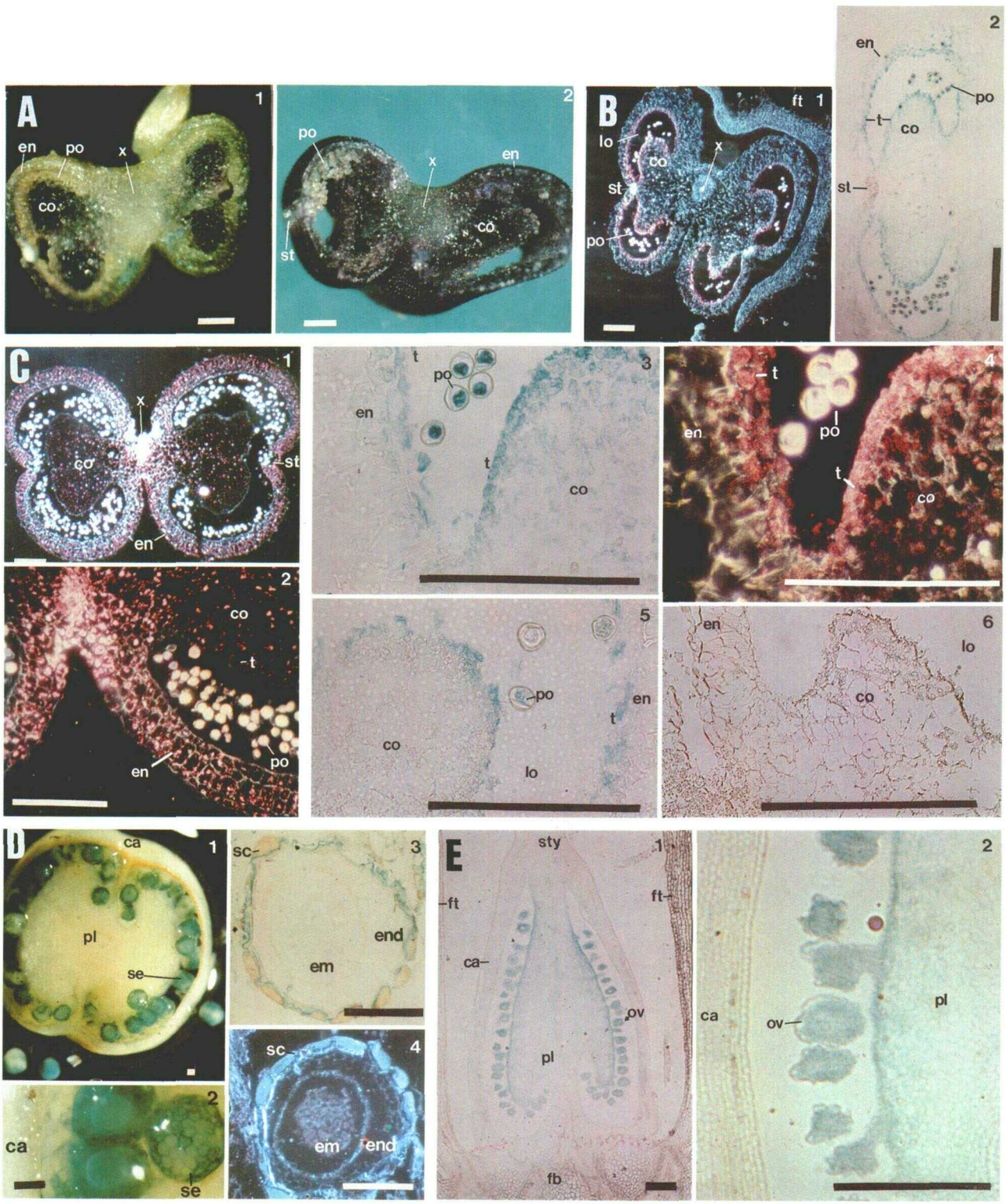

Figure 5. Histochemical Localization of Anthocyanins and GUS Activity in Reproductive Organs of Transformed Plants.

Tissue-Specific Accumulation of Flavonoids Suggests a Highly Differentiated Expression of Biosynthetic Genes

The activity of flavonoid biosynthetic genes in ovaries and seeds has not been examined before. To determine whether flavonoid biosynthetic genes themselves are active in these unpigmented tissues and which type of flavonoid is accumulated, we analyzed extracts of ovaries and seeds from several petunia varieties by thin-layer chromatography. Because all known petunia varieties contain mutations for at least one flavonoid biosynthetic gene, we used several different varieties with a well-defined genotype and included extracts of pigmented tissues as a control. Some of the relevant data are summarized in Table 1. In Figure 6 a simplified scheme is presented that shows the biosynthetic pathway of flavonols and anthocyanins in petunia flowers and the genes involved. In ovaries a variable amount of (colorless) flavonols was detected, depending on the variety, but never anthocyanins (Table 1). The amount of flavonols in the corollas, which are known to be dependent on the genetically defined gene *Fl* (Gerats et al., 1982), shows an identical pattern of variation. This suggests that *Fl* may also influence the flavonol content of the ovary. In lines dominant for one or more of the known hydroxylation genes (*Ht1*, *Ht2*, *Hf1*, and *Hf2*), the accumulated flavonol is quercetin, whereas lines recessive for all four of them (W80) accumulate kaemferol. This suggests that each of these genes is expressed in the ovary. In extracts of seeds the anthocyanin delphinidin is found in addition to very low amounts of flavonols, irrespective of the status of the hydroxylation, glycosylation (*Gf*), rhamnosylation (*Rt*), and methylation genes (*Mt1*, *Mt2*, *Mf1*, and *Mf2*).

Despite the presence of anthocyanins, young developing seeds are white; pigmentation becomes evident only after acidic extraction of flavonoids. Possibly, the accumulated anthocyanin is the (colorless) leuco-delphinidin, which is converted to delphinidin because of the acid treatment. Alternatively, the lack of pigmentation may be due to the way the anthocyanin is stored. The *An6* locus has been shown to contain the *dfr*A gene (Beld et al., 1989; H. Huits and A.G.M. Gerats, personal communication), encoding dihydroflavonol 4-reductase, the first enzyme from the anthocyanin pathway. In lines recessive for *an6* (W80 and W85), no anthocyanins were found in any of the tissues tested. This indicates that *dfr*A is the major expressed member of the *dfr* gene family in these tissues. The loci *An1* and *An2* contain regulatory genes of the pathway (Beld et al., 1989), but their action shows a different tissue specificity. Anthyocyanin accumulation in seeds and flower stems is dependent on *An1* but independent of *An2*.

DISCUSSION

In this paper we report a detailed analysis of the expression of four members of the *Petunia hybrida* (V30) chalcone synthase (*chs*) multigene family. Previous RNase protection experiments have shown that two members (*chs*A and *chs*J) of this gene family are active in the flower tube, corolla, and in UV-illuminated seedlings (Koes et al., 1989a). *chs*B and *chs*G are silent in normal plant tissues, but their expression can be induced by UV light in seedlings. Chimeric genes consisting of the promoter of each of these *chs* genes fused to the β-glucuronidase reporter gene (Figure 1) retained their tissue-specific expression pattern (Figure 3). This implies that the promoter region plays an important role in determining the different expression patterns of these *chs* genes, but does not exclude that other parts of these genes are also involved. These findings are in line with sequence data showing that the *chs*J and *chs*A promoters contain significant stretches of homology, but are completely different from the *chs*B and *chs*G promoter (Koes et al., 1989a). Experiments to iden-

Figure 5. (continued).

(A) Localization of anthocyanins in anthers from a stage-1 (section 1) and a stage-4 (section 2) flower of the variety V30.

(B) Localization of GUS activity in anthers from *chs*A-GUS transformants. Section 1: Dark-field micrograph of a stage 2 anther of a *chs*A-GUS transformant, stained with X-gluc. Section 2: Bright-field micrograph of a stage 2 anther of another *chs*A-GUS transformant, stained with X-gluc. Section 3: Detail of section 2. Section 4: Dark-field image of the section shown in section 3. Sections 5 and 6: Bright-field micrographs of sections of stage 4 and stage 6 anthers, respectively, of the same *chs*A-GUS transformant as in sections 2 to 4.

(C) Localization of GUS activity in stage-2 anthers of a CaMV-GUS-transformed plant. Section 1: Overview of a complete anther. Section 2: Detail of an anther.

(D) Localization of GUS activity in seedpods from a *chs*A-GUS transformant. Section 1: Macroscopical image of a stained seedpod. Section 2: Detail of the picture shown in section 1. Sections 3 and 4: Bright-field and dark-field micrographs, respectively, of a cross-sectioned single seed.

(E) Localization of GUS activity in ovaries from a *chs*J-GUS transformant. Section 1: Bright-field micrograph of a whole ovary. Section 2: Detail of section 1.

Note that GUS activity is represented by blue stain in bright-field micrographs and by red/pink stain in dark-field micrographs. Pollen and seed coat cells, however, do not stain red/pink in dark-field microscopy because of the structure of these cells. Scale bars equal 300 μm in all sections. Abbreviations: ca, capsule; co, connectivum; em, embryo; en, endothecium; end, endosperm; fb, flower base; ft, flower tube; lo, locule; pl, placenta; po, pollen grain; ov, ovule; st, stomium; sty, style; t, tapetum; x, xylem.

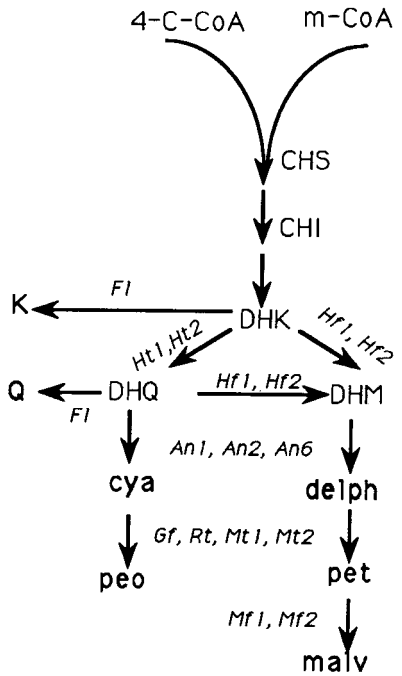

Figure 6. Simplified Scheme of the Flavonoid Biosynthetic Pathway in Flowers of Petunia.

The anthocyanins/flavonols are shown as far as they can be discriminated by TLC analysis (see Table 1): flavonols in bold capitals; anthocyanins in bold lower-case letters. Important intermediates are shown in normal capitals. For each series of reaction(s), the genes controlling these steps in the flower are shown in italics. The gene *Fl* controls the amount of flavonols synthesized, i.e., in *fl/fl* recessive lines the flavonol content is reduced 10-fold. For a more detailed description of the biochemistry and genetics of the pathway, see Schram et al. (1984), Wiering and de Vlaming (1984), and Gerats et al. (1987). Abbreviations: 4-c-CoA, 4-coumaroyl-CoA; cya, cyanidin; delph, delphinidin; DHK, dihydrokaemferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; K, kaemferol; malv, malvidin; 4-m-CoA, 4-malonyl-CoA; pet, petunidin; peo, peonidin; Q, quercetin.

tify regulatory elements in the *chs*A promoter will be presented elsewhere (van der Meer et al., 1990). In addition to organ specificity, the temporal control of expression during flower development appeared to be retained (Figure 1). The GUS mRNA levels in *chs*A-GUS transformants were remarkably low (in the highest expressors only few percent of the endogonous *chs* mRNA). Comparison of the expression levels of various chimeric genes driven by the *chs*A promoter or CaMV promoter suggests that an enhancing element present in the gene is absent in the *chs*A-GUS fusion. A more thorough discussion on this point will be published elsewhere (van der Meer et al., 1990).

Analyses of the expression of the *chs*A-GUS and *chs*J-GUS fusion at the cellular level revealed that promoter activity was often closely associated with pigmentation, although some discrepancies were found. In the tube of the flower, expression of both genes occurred mainly, but not strictly, in epidermal cells, coinciding with the site of anthocyanin accumulation. In the flower corolla, both fusion genes were expressed in both the epidermal cell layers and in the mesophyl cells in between, whereas the anthocyanins accumulated mainly in the epidermal cells. Because we observed this phenomenon for both the *chs*A-GUS and *chs*J-GUS fusions and for a *chi*A-GUS fusion (van Tunen et al., 1990), we consider it unlikely that it is caused by the absence of some *cis*-acting regulatory element in the promoters used. Moreover, experiments in which CHI enzyme activity was measured in stripped epidermal cells and residual fractions of corollas provide independent evidence that CHI enzyme activity is localized in epidermal as well as in mesophyl cells (Jonsson et al., 1984). Based on discrepancies between *chs* expression and flavonoid accumulation patterns in primary leaves of oat, it was postulated that intercellular transport of flavonoids may occur (Knogge and Weissenbock, 1986). It is possible that transport of anthocyanins or their precursors occurs from mesophyl cells to epidermal cells in the corolla of petunia. Alternatively, it might be that expression of *chs* and *chi* genes in mesophyl cells is related to synthesis of colorless flavonols.

In early stages of anther development, the anthocyanins were located in the parenchymal cells of the connectivum (Figure 5A). In these cells *chs* promoters were active (Figure 5B). No anthocyanins were detected in the exothecium even though *chs* promoters were also active in the tapetal and associated cells of the exothecium. It may be that activity of *chs* promoters in these cell types is related to synthesis of colorless flavonols. Such compounds are indeed present in anthers. In later stages of anther development, anthocyanins were evenly distributed throughout the anther (Figure 5A1). However, no mRNAs for the flavonoid biosynthetic enzymes can be found at these developmental stages (van Tunen et al., 1988; Beld et al., 1989; Koes et al., 1989a), which makes it unlikely that the genes are activated in other cell types. In addition we did not detect activity of *chs*-GUS genes in the outer cells of the exothecium or the stomium at any developmental stage, even though these cells become pigmented late during development. This again suggests that transport of anthocyanins or its precursors may occur.

Immunochemical studies of tulip anthers showed that the CHS enzyme is localized in the tapetum cells, whereas flavonoids appear to be localized at the outer surface of the pollen grain: the exine and/or its structures (Kehrel and Wierman, 1985). In this case it was suggested that flavonoid biosynthetic enzymes may be transported from tapetum cells to the free space of the locule. During maturation of the anther, the amount of GUS activity that was detectable by histochemical assay decreased (Figure 5B). How-

ever, the fluorimetric data showed that the amount of GUS activity remained constant (Figure 2). Because the contents of the anther locule contributed to GUS activity in fluorimetric assays but were lost after sectioning and X-gluc staining, this suggests that in late developmental stages (stage 6) GUS activity is present in the locules of the anther. This is in line with the observation that the GUS activity in stage 4 is not localized in cell structures but in small droplets that represent the remains of degenerating tapetum cells. Because anthers still contain high levels of flavonoid biosynthetic enzymes at the moment of tapetum degradation (van Tunen and Mol, 1987), it is reasonable to assume that these enzymes as well as the flavonoids accumulated are also liberated from the tapetal cells into the locule.

Recently, genes encoding dihydroflavonol 4-reductase (DFR, an enzyme acting later in the anthocyanin pathway, but not involved in flavonol synthesis) have been cloned from petunia (Beld et al., 1989). Analysis of the cell type-specific expression of these genes may shed more light on the differences between *chs* expression and anthocyanin accumulation patterns. Such experiments are in progress.

In addition to the tissues mentioned above, we found expression of both *chs*J-GUS and *chs*A-GUS in the ovary and after pollination in the seeds. In ovaries expression of *chs* genes appeared to be related to synthesis of flavonols and no anthocyanins were formed (Table 1). In seeds both anthocyanins and flavonols were found. The genetic control of flavonoid biosynthesis in seeds appears to differ considerably from that in the flower. Independent of the status of the hydroxylation, glycosylation (*Gf*), rhamnosylation (*Rt*), and methylation genes (*Mt1*, *Mt2*, *Mf1*, *Mf2*) involved in anthocyanin biosynthesis in the flower, (leuco) delphinidin was accumulated in seeds (Table 1). Anthocyanin accumulation in the seed was dependent on the regulatory gene *An1*, but independent of another regulatory gene, *An2*. A similar phenomenon was observed in maize for the regulatory genes *R* and *C1*. The *C1* locus affects anthocyanin synthesis in the aleurone layer of the kernel, whereas *R* affects the synthesis in a large number of plant tissues.

At present we can only speculate about the biological function of the flavonoids synthesized in the various tissues. It seems reasonable to assume that the anthocyanins accumulated in the corolla and tube of the flower are attractants for pollinators. This is in line with the observation that they are accumulated mainly in the inner epidermis of the flower. Flavonols, although not visible to the human eye, are visible to bees and, thus, may serve a similar function. It may be that the accumulation of flavonoids in anthers is also involved in attraction of pollinators. However, if this were the only function, one would not expect such a complex pigmentation and gene expression pattern as described in this paper. It has been shown that anthers from maize mutants, in which the synthesis of flavonoids in anthers is blocked, are sterile (Coe et al., 1981). This suggests additional functions for flavonoids in the development of anthers. To explain their accumulation in epidermal cells of young leaves, it has been suggested that flavonoids play a defensive role (Beerhues et al., 1988). Such a defensive role would be consistent with the expression site (seed coat) of *chs* genes in seeds. However, it does not explain the observed *chs* expression patterns in the ovary.

More conclusive data concerning the role of flavonoids may be obtained by preventing or modulating their synthesis. Recently, it was shown that flavonoid biosynthesis in the flower corolla could be completely or partially inhibited by the introduction of an antisense *chs* gene driven by the CaMV promoter, but pigmentation of anthers was unaffected (van der Krol et al., 1988). This is likely to be the result of the low activity of the CaMV promoter in the cell types expressing *chs* genes (parenchymous cells of the connectivum, Figure 5). This implies that flavonoid synthesis may be blocked in one cell type without affecting other cells if an appropriate promoter is used to drive expression of the antisense gene.

METHODS

DNA Methodology

DNA isolation, subcloning, restriction analyses, 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). Isolation of RNA and primer extension analysis were done according to published procedures (Koes et al., 1989b), using a synthetic 18-mer complementary to the 5′ end of the GUS coding sequence as a primer.

Construction of *chs*-GUS Fusion Genes and Transformation of Petunia Plants

chs-GUS-nos fusions were generated by cloning of promoter fragments of different *chs* genes in the pBI101 plasmids (containing a promoterless GUS coding sequence fused to a nopaline synthase 3′ end; Jefferson et al., 1987) in such a way that the ATG of the *chs* gene was in frame with the GUS coding region (translational fusions) as described below (see also Figure 1). Numbering of the sequences was relative to the transcription start (see Koes et al., 1989a, 1989b for details on sequences and clones used).

An NcoI site was created around the ATG translation start site of *chs*A by site-directed mutagenesis (VIP120; van der Meer et al., 1990). The *chs*A promoter plus the mRNA leader (from nucleotide [nt] −805 to +79) was isolated by digestion with EcoRI and NcoI, filled in with Klenow polymerase, and cloned in the HincII site to generate a HindIII site on the 5′ end (VIP120.01). After digestion of VIP120.01 with HindIII and BamHI, the *chs*A

promoter was fused in frame to the GUS gene of pBI101.1 digested with the same enzymes (VIP161, *chs*A-GUS).

To generate *chs*J-GUS, VIP160 (consisting of an XbaI/SstI fragment from VIP159 on which part of the first exon plus 1500 nt of promoter sequence was located, cloned in pTZ) was digested with BstEII (which cuts 1 nt downstream of the ATG), filled in with Klenow polymerase, digested with HindIII, and cloned into HindIII/SmaI-digested pBI101.2 to yield VIP 165 (*chs*J-GUS).

The two *chs*B-GUS fusions were constructed by digestion of VIP6724 (containing the complete *chs*B gene as a HindIII/XbaI fragment in pTZ) with AvaII (cuts 49 nt downstream of the ATG), filling in with Klenow polymerase, digestion with HindIII, and cloning into HindIII/SmaI-digested pBI101.2 and pBI101.3 to give VIP163 and -164, respectively.

*chs*G-GUS was generated by cloning a HindIII/BalI (HindIII cuts at −800 and BalI cuts 1 nt downstream of the ATG) fragment from VIP80.319 (a deletion derivative of VIP80 constructed by exonuclease III treatment) into HindIII/SmaI-digested pBI101.2.

Sequence analysis of the fusion sites was carried out using a primer complementary to the 5′ end of the GUS coding sequence to ensure that the *chs*-GUS fusion was in frame.

The constructs were transferred to *Agrobacterium tumefaciens* (LBA4404) by three-parental mating. Exconjugants were used to transform *Petunia hybrida* leaf discs as described by Horsch et al. (1985). After shoot and root induction on kanamycin-containing media, plants were put in the soil and kept in a greenhouse. Plants regenerated (on kanamycin-less media) from leaf discs treated with the LBA4404 strain without a binary vector served as a control for background GUS activity.

Fluorimetric GUS Assay

GUS activity in tissue extracts was measured fluorimetrically as described (Jefferson et al., 1987). Fluoresence values were corrected for quenching of the extract by measuring the increase in fluoresence after addition of a known amount of 4-methylumbelliferyl. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

Histochemical Localization of GUS Activity in Transformed Plants

Histochemical localization of enzyme activity is subject to a number of potential artifacts. For instance, cell size, penetration of substrate into the cell, background enzyme activity, and variable expression patterns in independent transgenic plants may all contribute to differences in staining intensity. To minimize these effects, we repeatedly performed assays on every type of organ/tissue, both in the same and in independent transgenic plants, and included negative (plants transformed without a binary vector) and a constitutive positive (CaMV-GUS) controls. We noted that the intensity of staining alone is an unreliable measure for levels of GUS activity because it quickly reaches a maximum plateau and, hence, tissues can be easily overstained. Therefore, we examined tissues after various periods of staining for more quantitative interpretations. Although we observed variation in the degree and kinetics of staining, the pattern of staining was reproducible.

For localization of GUS activity inside an organ, the organ was cut in large pieces with a razor blade and incubated for varying periods in 50 mM Na-phosphate buffer, pH 7, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Research Organics Inc.) at 37°C. This resulted in staining of cells up to 1 mm into the tissue, and could be examined with a stereomicroscope.

For analysis at the single cell level, X-gluc-stained tissues were fixated in 50 mM Na-phosphate buffer containing 1% glutaraldehyde and 1% formaldehyde for 16 hr and dehydrated by sequential passage through a series of ethanol solutions (1 × 70% for 4 hr to 16 hr, 1 × 80% for 30 min, 1 × 90% for 30 min, 1 × 96% for 30 min, and 1 × 100% for 30 min), amyl acetate/ethanol 1:1 (1 × 30 min), amyl acetate (1 × 30 min), and liquid paraffin (2 × 30 min at 50°C). After solidification of the paraffin (>16 hr at room temperature), 7-μm-thick sections were cut using a microtome and put on microscope slides with a drop of 1% protein glycerin (Merck). The slides were kept on a 45°C plate to flatten the sections and subsequently dried for 16 hr at 37°C. Finally, the sections were treated for 5 min with xylene to remove paraffin, mounted with malinol (Merck) and a coverslip, allowed to harden overnight, and examined by normal light- or dark-field microscopy. During the embedding procedure, the cytoplasm shrank and became visible after sectioning as stained droplets (blue in light-field, red in dark-field microscopy) inside the original cell wall.

Detection of Flavonoids

Tissues were incubated in 2 M HCl for 16 hr and after hydrolysis (20 min at 100°C) flavonoids were extracted in a small volume of isoamylalcohol and applied on cellulose TLC plates using acetic acid:hydrochloric acid:water (3:3:10) as the developing solvent.

To localize anthocyanins at the cellular level, complete flowers, or small sections thereof, were frozen in liquid nitrogen, freeze-dried, fixed in para-formaldehyde vapor, and embedded directly in liquid paraffin. After solidification 10-μm-thick sections were cut using a microtome and put directly on microscope slides. Paraffin was removed by carefully applying a drop of xylene on one of the edges of the sections. These slides were then examined directly using light- and dark-field microscopy.

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