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A cytochrome b_5 is required for full activity of flavonoid 3′,5′-hydroxylase, a cytochrome P450 involved in the formation of blue flower colors

(anthocyanin/*dTph1*/phenylpropanoid/blue gene)

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ABSTRACT **The substitution pattern of anthocyanin pigments is a main determinant of flower color. Flavonoid 3′,5′-hydroxylase (F3′5′H) is a cytochrome P450 enzyme (Cyt P450) that catalyzes the 3′,5′-hydroxylation of dihydroflavonols, the precursors of purple anthocyanins. Species such as rose and carnation lack F3′5′H activity and are, therefore, unable to generate purple or blue flowers. Petunia, on the other hand, contains two loci, termed *hf1* and *hf2*, that encode a Cyt P450 with F3′5′H activity. Here we report the identification of an additional petunia gene that is required for 3′,5′ substitution of anthocyanins and purple flower colors. It encodes a cytochrome b_5 and is expressed exclusively in the flower. Inactivation of the gene by targeted transposon mutagenesis reduced F3′5′H enzyme activity and the accumulation of 5′-substituted anthocyanins, resulting in an altered flower color. However, no phenotypic effect on the activity of other Cyt P450s, involved in the synthesis of hormones or general phenylpropanoids, was observed. These data provide *in vivo* evidence for the regulation of the activity of specific Cyt P450s by a cytochrome b_5.**

Cytochrome P450 (Cyt P450)-dependent monooxygenases are a large group of membrane-bound heme-containing enzymes that are involved in a range of NADPH- and O_2-dependent hydroxylation reactions. In animals Cyt P450s have been well studied because of their role in the metabolism of xenobiotic drugs and toxic chemicals as well as endogenous compounds, such as sterols, fatty acids, and prostaglandins (1). Plant Cyt P450s proved more difficult to purify. Only a limited number of plant Cyt P450 genes could be successfully purified and reconstituted (for examples, see refs. 2–4). As an alternative, genetic experiments were used to identify plant Cyt P450s involved in the synthesis of gibberellin (5), brassinosteroid (6), or yet unidentified hormones (7), as well as phenylpropanoids (8) and flavonoid flower pigments (9).

Cyt P450s depend for their activity on associated proteins such as a NADPH:Cyt P450 reductase, which catalyzes the transfer of electrons from NADPH via FAD and FMN to the prosthetic heme group of the Cyt P450 protein. Numerous studies have shown that the activity of Cyt P450s in reconstituted membrane vesicles (for examples, see refs. 10 and 11) or in yeast cells (12) can be enhanced by addition of cytochrome b_5 (Cyt b_5), suggesting that Cyt b_5 may act as an alternative electron donor. However, this stimulatory effect is not seen in all experiments (ref. 13, see also *Discussion*). Also, no clear consensus has emerged regarding the question by what mechanism Cyt b_5 influences Cyt P450 hydroxylation reactions

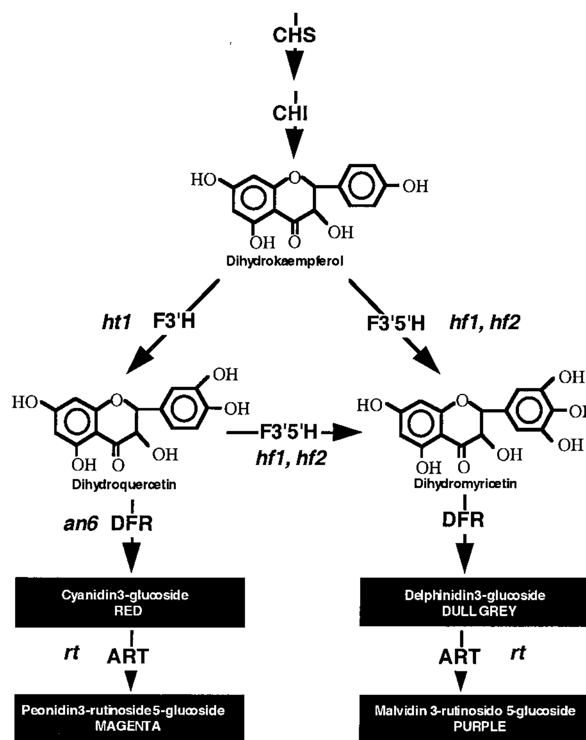

FIG. 1. Anthocyanin biosynthesis pathway in petunia. Enzymes are indicated in capital letters and the names of the genetic loci encoding these enzymes are indicated in italics. CHS, Chalcone synthase; CHI, chlacone-flavaonone isomerase; DFR, dihydroflavonol 4-reductase; ART, anthocyanin-rhamnosyltransferase.

(11–13). Genetic strategies can provide a powerful alternative to study the interactions and physiological functions of Cyt P450 and Cyt b_5 proteins under *in vivo* conditions.

In plants, the action of Cyt P450 enzymes involved in flavonoid synthesis is directly visible through the color of the flower. The hydroxylation of (colorless) dihydroflavonols in the 3′ and 5′ positions by Cyt P450 enzymes is a particularly important step, because this step determines whether red or purple/blue anthocyanins are formed (Fig. 1). In petunia, the

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Abbreviations: Cyt b_5, cytochrome b_5; Cyt P450, cytochrome P450, F3′5′H, flavonoid 3′,5′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; C4H, cinnamate 4-hydroxylase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF098510).

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ht1 locus, which is required for red and magenta flowers (14), encodes the Cyt P450 enzyme flavonoid 3′-hydroxylase (F3′H; F. Brugliera, personal communication). Two other loci, *hf1* and *hf2,* determine the substitution of anthocyanins in the 3′ and the 5′ positions and the generation of purple and blue flowers. Isolation of the *hf1* and *hf2* loci showed that both encode a Cyt P450 with flavonoid 3′,5′-hydroxylase (F3′5′H) activity (9). Plant species such as rose and carnation lack F3′5′H activity and are, therefore, unable to generate purple or blue flowers.

To identify additional genes involved in anthocyanin modification, we isolated cDNAs of eight genes or gene families (named *difA*, *difB*, *difC*, etc.) that are down-regulated in petunia flowers with a mutation in the regulatory *anthocyanin1* locus (15). Sequence analysis showed that *difA*, *difD*, and *difH* encode, respectively, anthocyanin synthase (16), chalcone synthase, and a sugar transferase, presumably UDP-glucose:flavonoid 3-glucosyltransferase or UDP-glucose:flavonoid 5-glucosyltransferase (unpublished data). Analysis of flower pigmentation mutants showed that *difG* originated from the *rt* locus and encodes anthocyanin-rhamnosyltransferase (15), whereas *difI* originated from the *an9* locus and encodes a glutathione *S*-transferase (17).

Here we show that *difF* encodes a Cyt b_5, a type of protein that was not previously implicated in the synthesis of flavonoids. Targeted inactivation of the *difF* gene by transposon insertion resulted in a flower color change caused by reduction of F3′5′H activity but did not have an apparent effect on other Cyt P450 enzymes. This result provides direct *in vivo* evidence that a Cyt b_5 protein stimulates the activity of specific Cyt P450s. Moreover, this finding suggests that *difF* may be a useful tool to boost the effectiveness of *f3′5′* in transgenic plants and introduce blue flower colors in ornamental species in which these are normally lacking.

MATERIALS AND METHODS

Molecular Genetic Procedures. Northern blot, PCR, and sequence analyses were done as described (18). The petunia lines W138 (relevant genotype: *an1-W138hf1⁻hf2⁻rt⁻*) and V30 (relevant genotype: *an1⁺hf1⁺hf2⁺rt⁺*) were maintained as inbred stocks and were grown under normal greenhouse conditions. Transposon insertion alleles of *difF* were isolated in the W138 background as described (19) with primers complementary to *difF* and *dTph1* and maintained by selfing. In the backcrosses of the *difF* mutant lines with V30 segregation of the unstable *an1-W138* and the linked *rt⁻* alleles were scored visually, whereas the anthocyanin substitution pattern was assayed by TLC (20) and in a few selected plants by HPLC (see below). Segregation of *hf1* and *hf2* alleles was determined by restriction fragment length polymorphism analysis (9) and by PCR amplification of the region containing the *dTph1* insertions for the mutant *difF* alleles.

HPLC Analyses and F3′5′H and C4H Assays. Total anthocyanins of flower corolla sectors were extracted and hydrolyzed by boiling in 1 ml of 2 M HCl for 30 min. The anthocyanin aglycones were analyzed on a gradient HPLC system equipped with a C_{18} reversed-phase column (5 μm; 250 × 4.6 mm; Vydac, Hesperia, CA) and a SPD-M10Avp diode array UV detector (Shimadzu). Samples were eluted at 40°C and a flow rate of 1 ml/min. Anthocyanins were monitored at 547 nm; dihydroflavonols were monitored at 280 nm. The solvent system used was a linear gradient of 10% to 75% solvent B (1.5% phosphoric acid/20% acetic acid/25% acetonitrile in water) in solvent A (1.5% phosphoric acid in water) over a period of 22.5 min. Anthocyanins were identified and quantified by comparison with the retention times and peak areas from standards. Microsomal membranes were isolated and assayed for F3′5′H activity as described (21), except that dihydroquercetin was used as a substrate and the formation of dihydromyricetin was monitored by HPLC and the solvent system described above. C4H activity was measured as described (22), and the conversion of cinnamic acid into 4-coumaric acid was monitored at 280 nm by HPLC, with the C_{18} column described above and 30% aqueous methanol with 0.1% acetic acid as a solvent.

RESULTS

***difF* Encodes a Cyt b_5 Protein.** Because the initial *difF* clone as isolated by Kroon *et al.* (15) was a partial cDNA, we isolated new *difF* cDNA clones to obtain the full cDNA sequence. We used primers complementary to the ends of the *difF* cDNA sequence and subsequently isolated the corresponding genomic regions by PCR. Comparison of the cDNA and genomic sequences showed that the *difF* mRNA is encoded by two exons (Fig. 2*A*). The *difF* cDNA sequence contained a single large ORF specifying a 149-aa protein. Database searches showed that this putative DIF-F protein had significant homology to a range of Cyt b_5 proteins from plants, animals, and yeast (Fig. 2 *B* and *C*). In all cases the homology was limited to the N-terminal half of the proteins, in particular in the regions around the residues His-39 and His-63. These His residues are known to be the axial ligands for heme binding

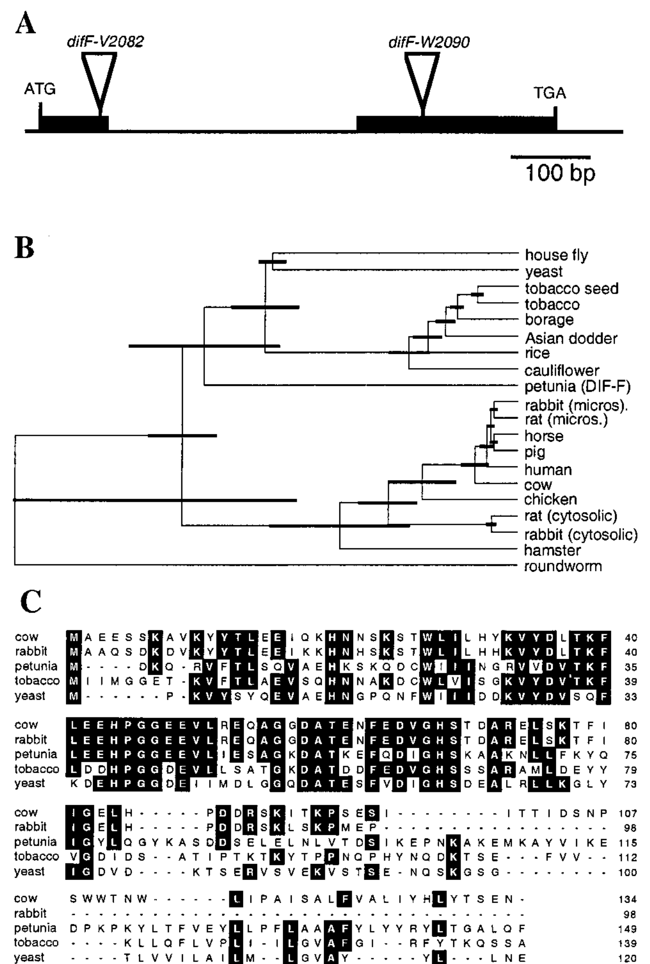

FIG. 2. Molecular analysis of *difF*. (*A*) Diagram showing the structure of *difF*. Exons are depicted as thick bars. The triangles indicate the positions of *dTph1* insertions in the alleles *difF-V2082* and *difF-W2090*. (*B*) Phylogenetic tree showing the homology of the DIF-F protein to a variety of Cyt b_5 proteins. (*C*) Alignment of the DIF-F protein with Cyt b_5s from mammals, plants, and yeast. Amino acids conserved in more than half of the sequences are indicated by black shading.

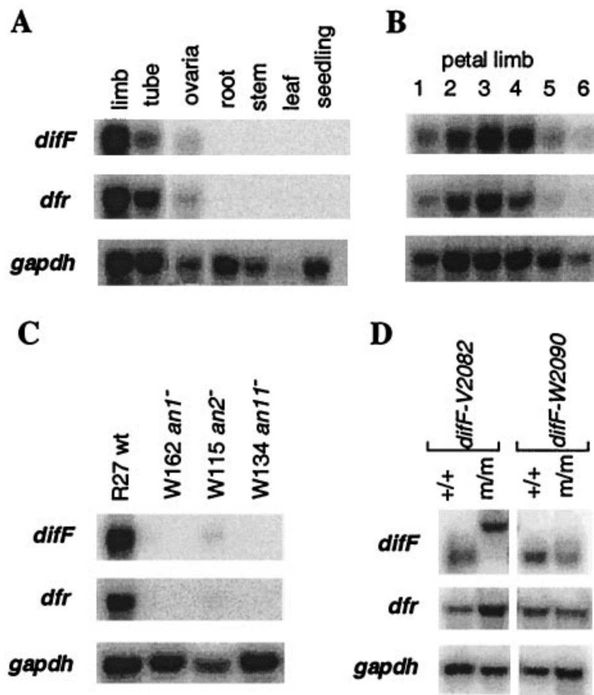

FIG. 3. Northern blot analysis of *difF* expression. *difF* expression in different tissues (*A*) and in the corolla limb at different stages (*B*, lanes 1–6) of development (*cf*. ref. 18). (*C*) *difF* expression in the corolla limb of wild-type (R27) and mutant lines (W162, W115, and W134) for the regulators *an1*, *an2*, and *an11*. (*D*) *difF* mRNA in corolla limbs homozygous for the mutable alleles *difF-V2082* or *difF-W2090* (m/m) and wild-type (+/+) siblings. gapdh, Glyceraldehyde-3-phosphate dehydrogenase.

(13). Although the Cyt b_5 sequences show little sequence conservation in the C-terminal part of the polypeptide, they have a strikingly similar hydropathy plot (not shown). This hydrophobic C-terminal part anchors the enzyme to the endoplasmic reticulum membrane (13).

***difF* Expression Pattern.** To examine whether *difF* could have a function in anthocyanin biosynthesis, we analyzed its expression pattern by Northern blot analysis and compared it to the expression pattern of the *dfr* gene, which encodes dihydroflavonol 4-reductase, a key enzyme of the anthocyanin pathway (23). Fig. 3*A* shows that the *difF* transcripts accumulate in the limb and tube of the flower corolla and in the ovaries, but not in vegetative organs such as leaves, root, and stem. During petal development, the temporal *difF* expression pattern closely matches that of *dfr*, with both transcripts reaching a maximum at approximately stage 3, when the flower bud starts to open (Fig. 3*B*). To test whether *difF* expression is controlled by any of the known regulators of the anthocyanin pathway, we analyzed *difF* transcript levels in stage 3 flowers of the corresponding mutants. Fig. 3*C* shows that *difF* expression is down-regulated in petal limbs of $an1^-$, $an2^-$, and $an11^-$ mutants, when compared with the wild type. Although *an2-W115* is a null allele (F. Quattrocchio, J. Wing, and R.K., unpublished data), this mutation reduces anthocyanin synthesis strongly but does not block it completely (24). This finding indicates that *an2* function is partially redundant and explains the residual *difF* and *dfr* transcripts detected in *an2-W115* petal limbs (Fig. 3*C*)

Taken together, these data show that the spatio-temporal and genetic control of *difF* expression are consistent with a role in anthocyanin synthesis.

Isolation of *difF* Mutants. To establish the *in vivo* function of *difF*, we isolated *difF* mutants. Therefore, we performed a PCR-based screen (19) to identify plants of the line W138 in which a *dTph1* transposon had inserted in the *difF* gene. Among 4,000 W138 plants, we found two individuals that were heterozygous for the wild-type $difF^+$ allele and a transposon insertion derivative (*difF-V2082* and *difF-W2090*, respectively). We germinated seeds of these individuals, which had been produced by self-pollination, and identified progeny homozygous for *difF-V2082* and *difF-W2090* by PCR. Sequence analysis showed that in *difF-V2082* a 284-bp *dTph1* element had inserted in the first exon, 10 bp upstream of the splice site, thereby disrupting the protein-coding sequence. The *difF-W2090* allele contained a 284-bp *dTph1* insertion in the middle of exon 2, which also disrupted the coding sequence. Northern analysis showed that flowers of *difF-V2082* homozygous progeny accumulated *difF* transcripts that were about 300 bp larger than the wild-type *difF* transcript (Fig. 3*D*). By analogy to other *dTph1* insertion alleles, this mutant transcript is likely to contain the transcribed *dTph1* sequence. In *difF-W2090* homozygotes, the amount of *difF mRNA* was reduced about 3-fold when compared with $difF^+$ siblings. Because *difF-W2090* is relatively unstable, these transcripts most likely resulted from *dTph1* excisions and probably contained different transposon footprints.

Mutation of *difF* Affects Flower Color. Despite the apparent inactivation of *difF*, we did not observe a phenotypic effect in the W138 genetic background. However, W138 is $hf1^-hf2^-$, and therefore, we could not examine an effect of the *difF* mutation on the activity of the Cyt P450 enzyme F3′5′H (compare with Fig. 1). To introduce the *difF* alleles in an $hf1^+$ or $hf2^+$ genetic background, we made backcrosses with the line V30 ($hf1^+hf2^+an1^+rt^+$) and the *difF* mutant lines as the recurrent parent. As expected, these progenies (co)segregated 1:1 for $an1^{\text{mutable}}$ and rt^- plants (Table 1). If the 5′ substitution of anthocyanin depended on the segregation of *hf1* and *hf2* alone, one would expect to find plants accumulating malvidin ($hf1^+hf2^+$ and $hf1^+hf2^-$), malvidin plus peonidin ($hf1^-hf2^+$;

Table 1. Number of plants with flower color and anthocyanins in the backcrosses (W138::$difF^{\text{mutable}}$ × V30) × W138::$difF^{\text{mutable}}$

	White with colored spots ($an1^{\text{mutable}}$)						Full colored ($an1^+$)					
	rt^+			rt^-			rt^+			rt^-		
Cross*	mal	mal/peo	peo	del	del/cya	cya	mal	mal/peo	peo	del	del/cya	cya
Z2363	← 44 →						← 49 →					
	← 4 →			← 39 →			← 48 →			← 1 →		
	3	0	0	15	14	7	16	22	3	0	1	0
Z2364	← 79 →						← 61 →					
	← 3 →			← 73 →			← 59 →			← 2 →		
	1	1	1	19	12	17	12	29	3	0	1	0
Total	← 121 →						← 110 →					
	← 7 →			← 112 →			← 107 →			← 3 →		
	4	1	1	34	36	24	38	51	6	0	2	0

Compounds: mal, malvidin; peo, peonidin; del, delphinidin; cya, cyanidin.
*Backcross family Z2363 segregated for *difF-V2082*, and Z2364 segregated for *difF-W2090*.

the relatively weak *hf2* locus enables the 5′ substitution of only about 50% of the anthocyanins), or peonidin ($hf1^{-}hf2^{-}$) corolla pigments in a ratio of 2:1:1. However, the combined results of the two backcross populations segregating for *difF-V2082* and *difF-W2090,* respectively, showed a segregation ratio of 38:51:6 (Table 1). This result suggested that a third mutant gene segregated that reduced the 5′ substitution, possibly *difF*. To test this directly, we subjected representative plants of the various phenotypic classes to Southern blot and PCR analyses to determine the *hf1*, *hf2* and *difF* genotype (results not shown). These experiments revealed that the malvidin-accumulating plants were all $hf1^{+}difF^{+}$, whereas those accumulating a mixture of malvidin and peonidin were either $hf1^{+}difF^{mutable}$, $hf1^{-}hf2^{+}difF^{mutable}$ or $hf1^{-}hf2^{+}difF^{+}$ (data not shown).

Closer inspection showed that the $hf1^{+}\,rt^{+}$ individuals, which were homozygous for the *difF-W2090* allele, had variegated flowers with purple (revertant) sectors and spots on a purplish magenta (mutant) background (Fig. 4*A Upper*). Also, flowers of $hf1^{-}hf2^{+}difF^{mutable}$ siblings were variegated, although the color difference between mutant and revertant tissue was less pronounced (not shown). In $hf1^{+}rt^{-}$ plants the variegation was seen as dull gray revertant spots and sectors on a dull red mutant background (Fig. 4*A Lower*). To test whether these variegated flower colors were caused by genetic instability of the *difF-W2090* allele, we isolated DNA from several large revertant petal sectors and from the mutant corolla sectors and analyzed the *difF* gene by PCR. Fig. 4*B* shows that reversions of the flower color are associated with (somatic) excisions of the *dTph1* element from *difF-W2090.* Also, *difF-V2068* individuals had variegated flowers, but the frequency of revertant spots was lower by at least 1 order of magnitude (not shown).

Mutation of *difF* Reduces 3′,5′ Modification of the Anthocyanin. To examine how the *difF* mutation affected flower color, we dissected (isogenic) $difF^{+}$ revertant and *difF* mutant sectors of single flowers and analyzed the anthocyanin aglycones by HPLC. Some representative chromatograms are shown in Fig. 4*C.* In $difF^{+}$ revertant petal sectors on $hf1^{+}rt^{-}$

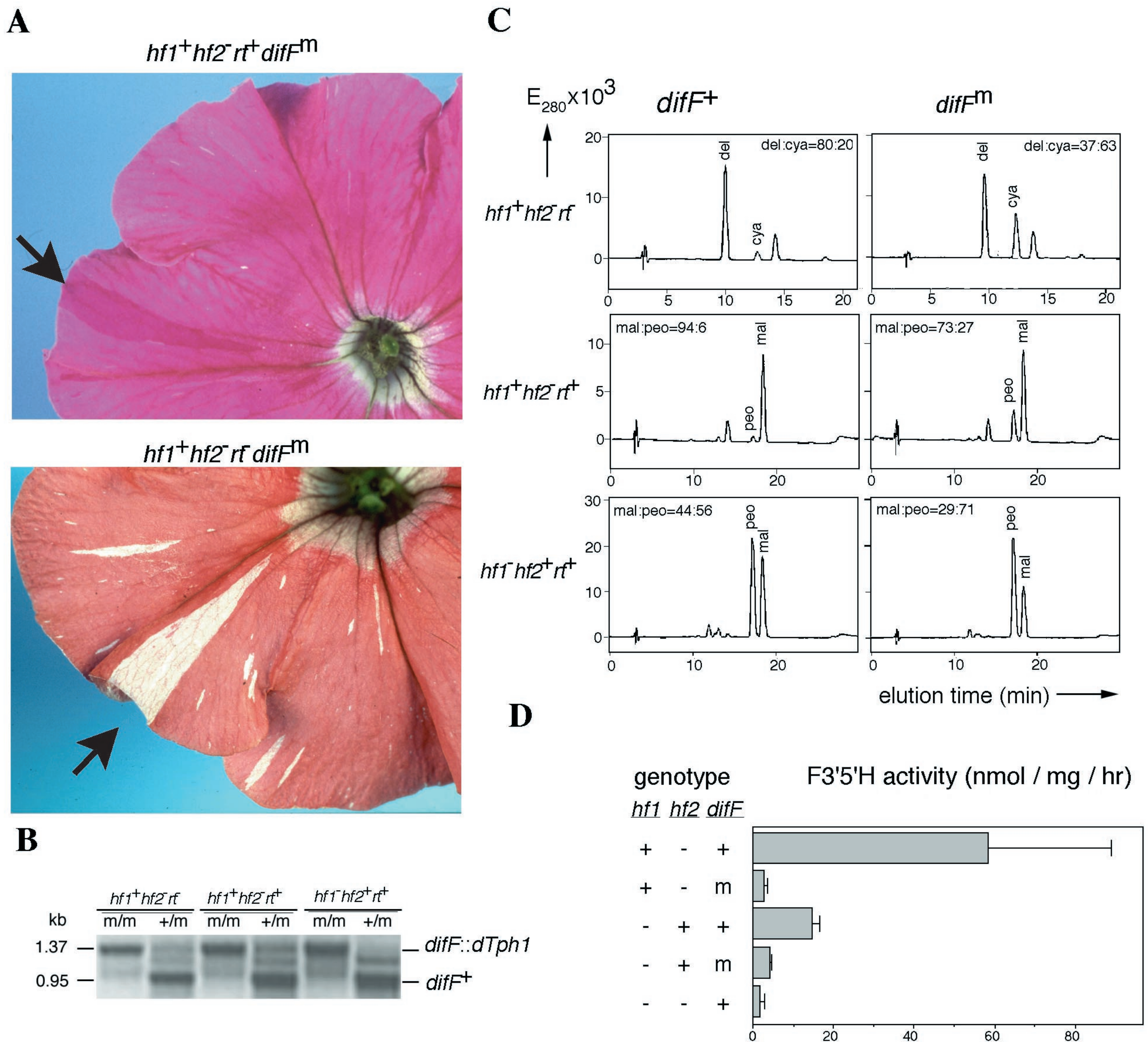

FIG. 4. Analysis of *difF* mutant flowers. (*A*) Phenotype of the *difF-W2090* allele in a $hf1^{+}rt^{+}$ (*Upper*) and a $hf1^{+}rt^{-}$ background (*Lower*). The arrows indicate a revertant ($difF^{+}$) sector. (*B*) PCR analysis of the *difF* locus in homozygous mutable (m/m) and revertant (+/m) sectors in flowers with different *hf1*, *hf2*, and *rt* genotypes. The intermediate size fragments are heteroduplexes consisting of a *difF::dTph1* and a $difF^{+}$ strand, which are generated by the PCR. (*C*) HPLC analysis of anthocyanin aglycones accumulated in the same sectors. The identity and the molar ratios of the anthocyanin peaks were established by chromatography of pure compounds: del, delphinidin; cya, cyanidin; peo, peonidin; mal, malvidin. (*D*) F3′5′H enzyme activity in the petal limbs of plants with the indicated phenotype selected from the backcross populations.

plants, about 80% of the anthocyanins were 3′,5′ substituted (delphinidin), whereas in *dif*^{mutable} mutant sectors of the same flower this amount was reduced to about 40% (Fig. 4*C Top*). The reduced delphinidin accumulation correlated with an increase in the accumulation of 3′-substituted anthocyanin (cyanidin) from 20% to 63%. This result indicates that the *difF* mutation reduced the formation of 3′,5′-hydroxylated anthocyanins by about 50% and that the remaining precursors were converted into a 3′-hydroxylated anthocyanin. The same phenomenon was observed in *hf1*$^{+}$*rt*$^{+}$ flowers. In this background, the *difF-W2090* mutation reduced the fraction of 3′,5′-substituted anthocyanins (malvidin) from 94% to 73%, which correlated with an increase in 3′-substituted anthocyanin (peonidin) from 6% to 27% (Fig. 4*C Middle*). This indicated a 25% inhibition in the formation of 3′,5′-substituted anthocyanins. In *hf1*$^{-}$*hf2*$^{+}$ tissue, less than half of the anthocyanins are 3′,5′ substituted (44%), possibly because the *hf2* locus expresses lower amounts of F3′5′H protein or a F3′5′H protein with lower activity. In this background, a *difF* mutation decreased 3′,5′ substitution further, down to 29%, corresponding to about 35% inhibition (Fig. 4*C Bottom*).

***difF* Mutation Reduces F3′5′H Activity.** To test whether *difF* stimulates 3′,5′ substitution of anthocyanin precursors by regulating the activity of the Cyt P450 enzyme F3′5′H, we measured F3′5′H activities in different genotypes. Because this required larger quantities of petal tissue, these measurements could not be performed on (isogenic) mutant and revertant sectors of single flowers. Instead, we selected two or three plants from the V30 backcross populations for each genotype and determined F3′5′H enzyme activity in microsomes that were isolated from stage 4 petal limbs. Fig. 4*D* shows that the *difF* mutation reduced *hf1*-encoded F3′5′H activity by about 20-fold, whereas *hf2*-encoded F3′5′H activity was reduced approximately 3-fold.

To test whether *difF* stimulates other Cyt P450 enzymes, we measured the activity of C4H, a Cyt P450 enzyme from the general phenylpropanoid pathway that synthesizes precursors for several pathways including the flavonoid pathway. Microsomal membranes of *difF*$^{+}$ and *difF*^{mutable} petals produced, respectively, 97.6 ± 11.2 and 84.0 ± 14.4 nmol of *p*-coumaric acid per mg of protein per h, indicating that the *difF* mutation did not have a significant effect on C4H activity.

DISCUSSION

Cyt b_5 proteins are common to animals, plants, and yeast and have been known for nearly 50 years. Yet we still know little about their physiological function. Biochemical experiments showed that *in vitro* Cyt b_5 can accept electrons from either NADH-cytochrome reductase or NADPH-Cyt P450 reductase and donate electrons to different desaturases involved in lipid biosynthesis and to members of the superfamily of Cyt P450s (reviewed in ref. 13). Here we provide *in vivo* evidence that the Cyt b_5 protein encoded by *difF* is required for full activity of the Cyt P450 enzyme F3′5′H *in vivo* and the generation of purple/blue flower colors.

Cyt b_5 Proteins and Cyt P450 Activity. Because Cyt P450 enzymes depend on associated proteins for their activity, purified Cyt P450s are usually incorporated into membrane vesicles together with other proteins to measure their activity. Not surprisingly, the NADPH-Cyt P450 reductase needs to be incorporated as well, because this protein donates electrons to the Cyt P450. In several *in vitro* studies the addition of Cyt b_5 could further stimulate Cyt P450 activity, suggesting that Cyt b_5 may function as an alternative electron donor. However, in many other studies, sometimes with the same proteins, no effect of Cyt b_5 was observed (discussed in ref. 13). Recent experiments showed that the stimulation of some Cyt P450s could also be achieved by an apo-Cyt b_5, devoid of the heme group (10, 12), or even unrelated Cyt P450s or Cyt P450 fragments (11). Oher Cyt P450s, however, could be stimulated only with holo-Cyt b_5, not with apo-Cyt b_5 (11). Because these results were all obtained with *in vitro* reconstituted systems, it is difficult to assess which effects are representative of the *in vivo* situation and which are experimental artifacts.

Cyt b_5 loss-of-function mutants would provide an excellent means to analyze the role of these proteins *in vivo*. In yeast, deletion of the *cyb5* gene did not display a phenotype, even though Cyt b_5 proteins were almost completely eliminated (25). However, in a yeast strain with a disrupted gene for NADPH:Cyt P450 reductase, the *cyb5* deletion was lethal (25). Although this result is consistent with the idea that Cyt b_5 is an alternative electron donor, this hypothesis could not be proven because it is unclear whether lethality is caused by reduced Cyt P450 activity or other malfunctions.

The Cyt b_5 Encoded by *difF* Is Required for F3′5′H Activity *in Vivo*. Our results show unequivocally that the *difF* gene augments the 3′,5′ substitution of (the precursors of) anthocyanins and is required for full F3′5′H activity. Plants in which the *difF* gene is reversibly inactivated by the insertion of a *dTph1* transposon bear variegated flowers, in which the accumulation of 3′,5′-substituted anthocyanins is diminished by 25–50%, apparently because of a reduction in the activity of the Cyt P450 enzyme F3′5′H (Fig. 4). These data imply that *in vivo* the *difF*-encoded Cyt b_5 enhances the activity of both F3′5′H isoenzymes encoded by the *hf1* and *hf2* locus (Fig. 4*D*).

For several genes encoding Cyt P450 enzymes, loss-of-function has been shown to result in visibly recognizable alterations. The activity of these Cyt P450 is apparently not substantially reduced in *difF* mutants, at least not to a the point where they become rate limiting, because we do not see these alterations in *difF* mutants. For instance, neither mutation of *difF* nor its regulators *an1*, *an2*, or *an11* (Fig. 3) has an effect on plant or organ shape, indicating that the activity of Cyt P450s involved in the synthesis of hormones (5–7) is not substantially reduced. Also F3′H activity appears to be normal in *difF* mutants. The line W138, in which the mutant *difF* alleles were isolated, is *hf1*$^{-}$*hf2*$^{-}$*ht1*$^{+}$*rt*$^{-}$. As a consequence, the *An1*$^{+}$ revertant spots on W138 petals are red because of accumulation of cyanidin (compare with Fig. 1). Mutation of *ht1* results in a strong reduction of cyanidin formation and pink spots and an accumulation of (colorless) dihydrokaempherol (20). Because the *difF* mutants do not exhibit either of these effects, F3′H activity apparently did not become rate limiting. The activity of C4H, a Cyt P450 involved in the synthesis of flavonoid precursors, also does not appear to be affected by *difF* because we did not detect a reduction of C4H enzyme activity or a reduction of flower color in *difF* mutants. Cyt b_5s have also been implicated as electron donors for desaturases involved in lipid biosynthesis (13, 26). However, we could not detect differences in the fatty acid composition of petals or seeds of wild types and *difF*$^{-}$ or *an1*$^{-}$ mutants (unpublished data).

Taken together these observations suggest that the Cyt b_5 encoded by *difF* is required for the activity of some Cyt P450s, such as F3′5′H, but not all. However, it does not exclude that (some of) these other Cyt P450s interact with Cyt b_5 proteins encoded by other loci.

Evolutionary Aspects. The regulatory genes *an1*, *an2*, and *an11* control expression of the so-called late biosynthetic genes encoding enzymes that are specific for anthocyanin synthesis. The early biosynthetic genes, encoding chalcone synthase, chalcone flavanone isomerase, and flavanone 3β-hydroxylase, which are required for anthocyanins, flavonols, and flavanones, are controlled by a different set of regulatory genes (24, 27). The *ht1* gene encoding F3′H is expressed independently of *an1*, *an2*, and *an11* (ref. 14; F. Quattrocchio and R.K., unpublished data), indicating that it is an early biosynthetic gene. However, the expression of the *f3′5′h* genes at the *hf1* and *hf2* loci (data not shown) and *difF* (Fig. 2) is controlled by

an1, *an2*, and *an11*, indicating that these are late biosynthetic genes. The distribution of various flavonoid classes over the plant kingdom indicates that the function of early biosynthetic genes arose first during evolution and that of the late biosynthetic genes and the anthocyanins appeared later (28). Thus, the function of F3′H might be evolutionarily older than that of *difF* and F3′5′H, which can explain why loss of *difF* function specifically reduces F3′5′H activity but not F3′H activity.

Applied Aspects. Many plant species lack the capability to generate blue or purple flowers, because they cannot synthesize 3′,5′-substituted anthocyanins. To introduce true blue and purple colors, *f3′5′h* transgenes have been introduced in ornamental species such as rose and carnation (*cf*. ref. 29). Both *in vitro* reconstitution experiments (13) and *in vivo* overexpression experiments in yeast (12) and human cells (30) have shown that the activity of a Cyt P450 can be increased 10- to 20-fold by coexpression of a Cyt b_5. Therefore, we expect that *difF* may be helpful to increase F3′5H activity in transgenic plants, which is considered the critical step toward the generation of blue flower colors in ornamental species (29, 31).

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