Genetic interactions underlying flower color patterns in *Antirrhinum majus*

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Diverse spatial patterns of flower color in *Antirrhinum* can be produced by a series of alleles of *pallida*, a gene encoding an enzyme required for pigment biosynthesis. The alleles arose by imprecise excision of a transposable element, Tam3, and we show that they carry a series of deletions involving progressive removal of sequences adjacent to the excision site. This has enabled us to define three *cis*-acting upstream regions, A, B, and C, which differentially affect the level of *pallida* expression in distinct areas of the flower. We show further that an unlinked locus, *delila*, regulates the spatial distribution of *pallida* transcript. Deletion of regions ABC at the *pallida* locus uncouples *pallida* from regulation by *delila*, whereas deletion of A or AB brings *pallida* under regulation by *delila* in a new area of the flower. These results suggest that diverse patterns of *pallida* expression reflect the different ways in which alleles interact with a prepattern of both common and spatially specific genetic signals in the flower.

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The molecular mechanisms by which multiple alleles at a single locus confer diverse phenotypic patterns are poorly understood. In most cases analyzed, the alleles are of genes encoding products that do not readily diffuse between cells. Diverse patterns are thought to be the result of differences in the way alleles interact with or interpret pre-existing patterns, termed prepatterns (Stern 1954, 1968). Molecular analysis of this interaction has been difficult because the genetic determinants of the prepattern are unknown in many of the cases, such as *yellow* or *scute* in *Drosophila*, where different alleles have been isolated (Campuzano et al. 1985; Chia et al. 1986). Furthermore, many of the alleles carry transposon insertions, confounding the regulation of the host gene with that of the transposon (Geyer et al. 1988). Perhaps the best study to date has been on the *white^{spotted}* alleles in *Drosophila*, which change the distribution of pigment between testis and eye and uncouple *white* from inhibition by the *zeste¹* mutant allele (Davison et al. 1985; Pirrotta et al. 1985). Here, we investigate the structure and expression of a series of alleles of the *pallida* (*pal*) locus conferring diverse patterns of flower color in *Antirrhinum majus* and how they interact with a regulatory gene *delila* (*del*). A particular advantage of studying the *pal* alleles is that they arose by imprecise excision of

a transposon, permitting a systematic study of a regulatory region of the *pal* gene, independently of the transposon (Coen et al. 1986).

The *pal* gene encodes a product required for the synthesis of red anthocyanin pigment in flowers (Stickland and Harrison 1974). Plants in which the expression of *pal* is completely blocked have ivory, rather than full red, flowers. An unstable recessive allele, *pal-2* (*pallida^{recurrens}*), carries the transposable element Tam3 inserted near the *pal* promoter, preventing *pal* transcription (Martin et al. 1985; Coen et al. 1986). Somatic excision of Tam3 during flower development gives rise to clones of pigmented cells on an ivory background, indicating that *pal* acts in a cell-autonomous manner. Excision of Tam3 in the germ line produces stable alleles, which give either the wild-type phenotype (full red) or altered intensities and patterns of pigmentation (Baur 1924; Fincham and Harrison 1967). Analysis of some of these *pal* alleles has revealed that they have nucleotide sequence alterations at the site of Tam3 excision (Coen et al. 1986).

Here, we describe a set of *pal* alleles with distinct patterns of expression that result from a series of deletions involving progressive removal of sequences adjacent to the Tam3 excision site. We show that an unlinked locus, *del*, regulates the spatial distribution of *pal* transcript. The *pal* deletions either uncouple *pal* from regulation by *del* or alter the spatial specificity of the *del* effect. The resulting patterns of *pal* expression can be explained by

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the different ways in which the alleles interact with a prepattern of both common and spatially specific genetic signals in the flower.

Results

Patterns of pal expression

The *pal* gene encodes dihydroflavonol-4-reductase, an enzyme that catalyzes the reduction of dihydroquercetin to leucocyanidin, a late step in the anthocyanin biosynthetic pathway (Kristiansen 1984; Heller et al. 1985). This was concluded from precursor feeding experiments in which pigment synthesis was observed when flowers of *pal* mutants were imbibed with leucocyanidin but not when they were supplied with dihydroquercetin. As a

control, flowers of a mutant at *incolorata* (*inc*), a gene that acts at an earlier step than *pal* in the anthocyanin pathway (Stickland and Harrison 1974; Forkmann and Stotz 1981), were imbibed with either leucocyanidin or dihydroquercetin; in each case, pigment synthesis resulted. Further evidence for the biosynthetic role of the *pal* product comes from the homology between the sequence of amino acids encoded by the first exon of *pal* and that of the A1 gene of maize which also encodes dihydroflavonol-4-reductase (Coen et al. 1986; Schwarz-Sommer et al. 1987).

Most of the stable alleles derived from the unstable allele *pal-2* confer the wild-type phenotype (Fig. 1; *pal-501*), in which, during flower development, anthocyanin synthesis occurs first in a ring at the base of the

Figure 1. Phenotypes conferred by various *pal* alleles (501, 33, 32, and 15) and a mutation at the *del* locus, at several stages of flower development. Alleles conferring similar phenotype to *pal-33* include *pal-518* and *pal-35G*. The *pal-15* allele confers a similar phenotype to *pal-569*.

tube and in the lobes (Fig. 1; *pal*-501, stage b). In the next stage (c), the tube becomes fully pigmented, giving a pattern that is maintained through to the final stage of development (d). Thus, anthocyanin synthesis appears to follow a temporal and spatial pattern that defines three areas in the flower corolla: the base of the tube, the area of the tube in which pigment synthesis occurs at a late stage, and the lobes. The *pal* alleles that confer different relative intensities of pigmentation in these three areas can be divided into three classes. The first class includes *pal*-15 and *pal*-569, which give color confined to the ring at the base of the tube that appears at stage b, the rest of the corolla remaining unpigmented (Fig. 1). The second class contains *pal*-32, which gives palely colored tubes and almost unpigmented lobes. This type of pattern is of particular interest because it is the inverse of that produced by mutations at the *del* locus, which is unlinked to *pal* and gives flowers with red lobes and unpigmented tubes (Wheldale 1907; Fig. 1). Alleles of the third class (*pal*-33, *pal*-35G, and *pal*-518) give pale color to the lobes and almost wild-type pigmentation in the tubes (Fig. 1; Table 1).

It was shown previously that alleles of all three classes (*pal*-15, *pal*-32, *pal*-33, and *pal*-518) produce reduced overall levels of *pal* transcript relative to wild type (Coen et al. 1986). Two explanations may be proposed for how changes in *pal* transcription can result in altered patterns of color. One is that diverse pigmentation patterns reflect changes in the spatial distribution of *pal* transcript. The other is that different threshold levels must be reached in distinct areas of the flower before a change in transcription results in altered pigment production. In this case, overall reductions in *pal* transcription without change in its spatial distribution could result in different patterns of color.

To determine the spatial distribution of transcript conferred by diverse *pal* alleles, Northern and slot-blots of RNA extracted from different areas of flower buds were probed with a *pal* clone. More *pal* transcript was detected in the lobes than in the tubes in *pal*-501 and *pal*-520, whereas less transcript was produced in the lobes than in the tubes in *pal*-518, *pal*-33, and *pal*-32

(Table 1; Fig. 2a,b). The quantity of *pal* mRNA at the base of the tube of *pal*-15 was reduced to ~1% of that in the *pal*-520 control in the same area (Fig. 2c). No transcript was detected in the remainder of the corolla for *pal*-15, whereas higher levels of *pal* mRNA were found in the rest of the corolla than at the base of the tube in *pal*-520 (Fig. 2c). These results show that distinct patterns of color conferred by the *pal* alleles reflect different spatial distributions of *pal* mRNA. As a control, the same blots were probed with a clone of the *nivea* (*niv*) gene that encodes chalcone synthase, an enzyme also required for anthocyanin synthesis (Wienand et al. 1982). The levels and spatial distributions of *niv* transcript were similar, irrespective of *pal* genotype (Table 1; Fig. 2). In another control, the relative levels of *pal* and *niv* transcripts produced in the upper and lower halves of the corolla in flowers from the lines containing *pal*-501 or *pal*-518 were determined. Transcripts of *pal* and *niv* were found to be distributed evenly between the two areas in both lines (Fig. 2d). Transcription initiation was also investigated to determine whether *pal* mRNA detected in blots was accurately initiated. S1 mapping of RNA produced by *pal*-501, *pal*-518, *pal*-33, *pal*-32, and *pal*-15 revealed that the sites of transcription initiation in all of these alleles were identical to those found previously in *Pal*⁺ and other mutant alleles (Coen et al. 1986; Robbins et al. 1989; data not shown).

Structures of *pal* alleles

It has been shown previously by Southern blots that many of the *pal* alleles arose by imprecise excision of Tam3 from the *pal*-2 allele (Coen et al. 1986). The regions around the excision site were sequenced for each allele (Fig. 3). In *pal*-501 and *pal*-520, both of which confer wild-type phenotype, sequence changes were confined to nucleotides of the 5-bp target duplication of *pal* sequence that flanked Tam3 in the *pal*-2 progenitor. In *pal*-520, 4 bp of each of the 5-bp target copies was retained and flanked a 4-bp inverted duplication of target sequence. The net results of these changes were insertions of 1 bp in *pal*-501 and 7 bp in *pal*-520, relative to

Table 1. Levels of *pal* and *niv* mRNAs in tubes and lobes of flowers carrying the *pal* alleles indicated

Allele	Anthocyanin			mRNA					
				<i>pal</i>			<i>niv</i>		
	T	L	T/L	T	L	T/L	T	L	T/L
501	100	440	0.25	100	200	0.5	100	420	0.24
520	110	470	0.25	130	190	0.7	130	400	0.32
518	100	130	0.80	25	20	1.2	120	430	0.28
33	60	50	1.20	25	10	2.5	160	420	0.37
32	10	5	2.00	4	1	4.0	160	420	0.37

All measurements are relative to the levels detected in the tubes of *pal*-501, which confers wild-type phenotype. T/L is the ratio of the levels in tubes and lobes. Alleles *pal*-501, *pal*-520, and *pal*-518 are maintained in isogenic backgrounds because they were derived by selfing a common progenitor containing *pal*-2, which has been inbred for many generations. Allele *pal*-518 had previously been considered to have a uniform reduction in pigmentation relative to wild type. However, as shown here and in Fig. 3, the level of pigmentation in the lobes is much more reduced than in the tubes. Alleles *pal*-33 and *pal*-32 were selected in progenies of crosses between lines containing *pal*-2 and other lines carrying mutant *pal* alleles. The S.E.M., based on five independent measurements, is 10–20% for the anthocyanin levels shown. The levels of *pal* and *niv* mRNAs were determined by slot-blot analysis.

Figure 2. Spatial distribution of *pal* and *niv* transcripts in flowers containing the *pal* alleles. Numbers above the blots indicate the *pal* alleles analyzed. Northern blots were first probed with a clone of the *pal* gene and then with a clone of *niv*. RNA was from flowers dissected as represented in the diagrams shown above the blots. The areas from which RNA was extracted are indicated above each lane: (T) Tubes; (L) lobes; (UH) upper half of the corolla; (LH) lower half of the corolla; (BT) base of the tube; (RC) rest of the corolla. The high intensity of the signal produced by *pal*-520 (in *c*) does not allow the differences in the levels of *pal* mRNA in BT and RC to be seen. A shorter exposure and slot-blot analysis revealed that in this case, the intensity of the signal obtained in RC was twofold higher than that in BT.

wild-type (*pal*-18). This suggests that normal levels of *pal* expression are compatible with the disruption of the Tam3 target sequence and small increases in the spacing between the regions to its left and right.

The three types of phenotypes conferred by mutant alleles correlate with progressive deletion of three regions to the left of the Tam3 excision site. Compared with *pal*-501, alleles *pal*-33, *pal*-35G, and *pal*-518 carry deletions of 7, 13, and 14 bp. The similar phenotype conferred by these alleles suggests that it results from deletion of a common 7-bp sequence, region A (Fig. 3). Dele-

tion of region A and a further 9–10 bp to its left (region B), results in reduced expression of *pal*-32. Deletion of regions AB, together with an additional 79 bp to the left (region C), results in expression confined to the base of the tube (*pal*-15). Removal of 21 bp to the left of region C (in *pal*-569) has no further effect on the pattern of *pal* expression.

Although the mutant alleles described fall into three classes, there are minor differences in phenotype within a class (e.g., *pal*-33, *pal*-35G, and *pal*-518; Fig. 3). This raises the question of whether some of this variation is the result of the small differences in sequence between these alleles or to the genetic backgrounds of the lines in which they are maintained. Analysis of an F₂ population from a cross between the lines containing *pal*-33 and *pal*-518 showed that flower color varied in the range defined by the parental lines. Because *pal*-33 and *pal*-518 can be distinguished on Southern blots (see legend to Fig. 3), the genotypes of several plants from this F₂, showing similar phenotypes, were determined. This revealed that flower color did not correlate with the combinations of the two alleles, suggesting that the differences in phenotype between the parental lines were caused by differences in genetic background. This was further confirmed by F₂ populations from intercrosses between lines carrying *pal*-501, *pal*-518, *pal*-33, *pal*-32, and *pal*-15. Phenotypes typical of the *pal*-518 parental line were often observed in crosses involving *pal*-33 and vice versa. Phenotypes typical of the *pal*-501, *pal*-32, and *pal*-15 parental lines were only observed when the corresponding alleles were used in the crosses, showing that genetic background was not responsible for the major phenotypic differences between the three classes of alleles.

The deletions and the inverted duplications in the various alleles can be readily explained by a hairpin model for the excision of Tam3 detailed elsewhere (Coen and Carpenter 1988; Coen et al. 1986, 1989). However, in *pal*-15, the mechanism by which a sequence normally located 125 bp upstream of the Tam3 target was directly duplicated at the junction of a 98-bp deletion and an inverted duplication remains unclear.

del regulates *pal* expression

Mutations at *del* give flowers with fully pigmented lobes and unpigmented tubes (Fig. 1). This is most probably the null phenotype because several independent *del* mutants, some of which are the result of transposon insertion, confer the same phenotype (R. Carpenter and E.S. Coen, unpubl.). To determine whether genes encoding enzymes of the pigment pathway were regulated by *del*, Northern and slot-blots of RNA from tubes or lobe of *Del*⁺ or *del* flowers were probed with clones of either the *pal* or *niv* genes. The levels of mRNA from both genes in the lobes were unaffected by *del* (Fig. 4). However, the quantity of *pal* transcript was reduced ~100-fold and that of *niv* ~5-fold in the tubes of *del* relative to *Del*⁺ flowers. These results show that the *Del*⁺ gene product is specifically required in the tubes but not in

the lobes for wild-type levels of *niv* and *pal* expression. Mutations at *del* also reduce the levels of *inc* transcript in flower tubes but have no effect in the lobes (C. Martin, unpubl.). In addition, the *del* gene regulates

genes acting at other steps in the anthocyanin pathway because imbibition of flowers mutant for both *pal* and *del* with leucocyanidin resulted in additional pigment synthesis in the lobes but not in the tubes. This is con-

Figure 3. Structure of *pal* alleles. (Top) The *pal-2* allele, which carries the transposable element Tam3. The wild-type allele, *pal-18*, comes from a commercial fully red line (see Coen et al. 1986). All other alleles shown have resulted from Tam3 excision. Nucleotides of the target duplication generated following integration of Tam3 in *pal-2* are represented by uppercase letters with horizontal arrows above. The sequences flanking these nucleotides are indicated by lowercase letters. Horizontal lines represent sequences that are identical in all alleles. For ease of illustration, 73 bp of sequence (dashed line) has not been shown in full. Open boxes represent deletions. Numbers inside the boxes indicate the sizes of the deletions (in bp) extending from the left end of the Tam3 target. The left end points of these deletions are indicated by vertical arrows on the sequence of *pal-2*. Solid squares in inverted duplications represent a nucleotide missing at the center of symmetry of what would otherwise be a perfect palindrome. In *pal-33*, the dinucleotide GG, indicated as part of an inverted duplication, might have been represented as part of the normal *pal* sequence, 8 bp to the left of the target. In this case, the deletion end point would have been 2 bp to the right of the site indicated. Horizontal dashed arrows indicate a sequence duplicated in direct orientation in *pal-15*. (Top) Solid bars represent regions A, B, and C (see text for details). Numbers above these boxes indicate their positions relative to the most upstream *pal* transcription initiation site. The sequences shown were consistent with a number of differences between the alleles revealed by Southern analysis of genomic DNA. Alleles *pal-520*, *pal-33*, *pal-32*, and *pal-15* carry the BstEII site (GGTNACC) generated by an inverted duplication of target nucleotides. The CCGG site, also generated at the Tam3 excision site in *pal-520*, was cleaved by HpaII. A NruI site, 10 bp to the left of the Tam3 target (TCGCGA), was present in *pal-33* and *pal-520* but not in *pal-35G*, *pal-32*, *pal-15*, or *pal-569*. In *pal-15* and *pal-569*, the HindIII fragments containing the Tam3 excision site were ~100 bp smaller than those from other alleles. The sequences of *pal-2*, *pal-18*, *pal-501*, and *pal-518* were reported previously (Coen et al. 1986).

Figure 4. Effect of *del* on *pal* and *niv* expression. A Northern blot of total RNA extracted from the tubes or lobes of *Del*⁺ or *del* flowers was probed with *pal* and *niv*, as described in Fig. 2. *Del*⁺ flowers were from a standard wild-type line (J17). The *del* allele is from line J18, which has an otherwise wild-type genotype.

sistent with the observation that the activity of UDP-glucose : flavonoid-3-O-glucosyltransferase, an enzyme acting at a later step than dihydroflavonol-4-reductase, can be detected in the lobes but not in the tubes of *del* flowers (Martin et al. 1986).

Interaction between *pal* and *del*

The observed patterns of expression in different *pal* alleles may reflect altered interactions between the alleles and regulatory genes that affect *pal* expression in a spatially specific manner (Coen and Carpenter 1986). To investigate whether the *pal* alleles showed an altered interaction with the regulatory gene *del*, their expression was analyzed in a *Del*⁺ or *del* background. Because *del* regulates several steps in the pigment pathway, *pal* expression in tubes of *del* mutants will not result in pigment production. This allowed *del* mutants to be recognized easily by the lack of tube pigmentation, irrespective of the *pal* genotype (Fig. 5) but precluded the use of pigmentation as an assay for *pal* expression in the tube.

To determine the effect of the *pal* deletions on the interaction between *pal* and *del*, the levels of *pal* transcript were measured in tubes and lobes of *del* and *Del*⁺

genotypes. Transcript levels were reduced in the tubes of *pal-32;del* relative to *pal-32;Del*⁺ flowers, indicating that *pal-32*, which lacks regions AB, was still regulated by *del* (Fig. 6a). Interestingly, *pal-32* expression in lobes was also clearly reduced in *del* mutants, resulting in complete abolition of pigmentation in lobes (Figs. 5 and 6a). This is in contrast to *Pal*⁺, *Niv*⁺, and *Inc*⁺ alleles, the expression of which is not affected by *del* in the lobes (Figs. 4 and 6). The effect of *del* on the lobes was also observed in flowers containing *pal-33* or *pal-35G* in which only region A is deleted. In these cases, *del* caused a threefold reduction in pigmentation levels in the lobes (Fig. 5). This indicates that in the absence of regions A or AB, the spatial specificity of the effect of *del* on *pal* expression is altered.

In *pal-15*, which lacks regions ABC, the levels and spatial distributions of transcript were found to be similar, irrespective of the *del* genotypes (Fig. 6b; see also Fig. 2c). As a control, we showed that *inc* was regulated by *del* in *pal-15* genotypes. This indicates that region C is required for the interaction between *pal* and *del*.

A previously described allele conferring a complex pattern of pigmentation, *pal-41*, also lacks regions ABC, because the entire region upstream of -70 has been re-

Figure 5. Phenotype conferred by *pal* alleles in a *Del*⁺ or *del* background. Note that the lack of anthocyanin in the tubes of the *del* mutants does not reflect the level of *pal* expression (see text and Fig. 6). The difference in lobe pigmentation between *pal-32;Del*⁺ and *pal-32;del* can be observed more easily at an earlier stage of flower development (stage c, Fig. 1).

Figure 6. Effect of *del* on the expression of *pal* alleles. Northern blots probed with *pal* and *inc* or *niv* are shown. RNA was from flowers containing the *pal* alleles indicated, in a *Del*⁺ (*D*⁺) or *del* (*d*) background. The areas of the flowers from which RNA was extracted are indicated above each lane (for details, see Fig. 2). (a and b) Each lane contains 3 grams of poly(A)⁺ RNA; (c and d) 2 μ g of total RNA was used.

placed by a new sequence as a result of a large-scale chromosomal inversion (Robbins et al. 1989) (Fig. 5). This allele produces ~25% of the level of transcript produced by *Pal*⁺. The levels of *pal* transcript at the base of the tubes and lobes in flowers containing *pal*-41 were similar in *Del*⁺ or *del* backgrounds (Fig. 6c,d). As expected, the control showed that *niv* was regulated by *del* in *pal*-41 genotypes. These results show that *pal* expression in the tubes and lobes of *pal*-41 is independent of *del* and confirm that region C is required for the interaction between *pal* and *del*.

Discussion

We have shown that different patterns of expression conferred by the *pal* alleles reflect the action of three *cis*-acting upstream regions, A, B, and C. Region A is a 7-bp sequence containing the palindrome CACGTG,

which is identical to the well-characterized upstream regulatory element of the adenovirus-2 major late promoter (Yu and Manley 1984; Carthew et al. 1985) and to the core sequence of the 'G' box, which has been found in several plant genes and shown to bind a nuclear factor (Giuliano et al. 1988; Shulze-Lefert et al. 1989). A protein specifically binding to this hexamer has also been described in *A. majus* (D. Steiger, H. Kaulen, and J. Schell, pers. comm.). Region B is 9 bp long and contains the sequence TTCGCG, which is identical to the core of the upstream element of the adenovirus-5 E1A-E promoter (SivaRaman and Thimmappaya 1987). Region C is 79 bp long and includes the CAAT box (Coen et al. 1986). The effects of these regions on expression, taken together with their structures and position, suggest that they most probably bind transcription factors. Similar multiple *cis*-acting upstream regions have been identified for many other genes by in vitro mutagenesis and transformation. However, unlike the analysis presented here, these studies assay gene expression in an abnormal chromosomal environment and subtle differences in pattern expression are not easily addressed (Fluhr et al. 1986; Dean et al. 1988; Willmitzer 1988).

The altered patterns of expression produced by deletion of the A, B, and C regions suggest that they interact directly or indirectly with a prepattern of spatially distributed genetic signals. To determine the nature of these interactions, we analyzed how the deletions affected regulation of *pal* by a *trans*-acting gene, *del*. In *del* mutants, the level of *pal* expression in the tubes is reduced to 1% of wild type, but the level in lobes is unaffected. In affecting expression of anthocyanin genes in particular regions, *del* resembles the *cl* locus of maize, which is thought to encode a transcription activator controlling gene expression only in the aleurone and scutellum (Chen and Coe 1977; Cone et al. 1986; Paz-Ares et al. 1987). We have shown that deletion of A, B, or C can alter the interaction between *pal* and *del* in two different ways. First, the deletion of A or AB brings *pal* under the regulation of *del* in the lobes. Second, deletion of ABC uncouples *pal* from regulation by *del*. How do these observations help to explain diverse patterns of *pal* expression resulting from the deletions?

The *pal* alleles that lack regions A or AB are regulated by *del* in the lobes as well as the tubes, suggesting that the *Del*⁺ gene product is present in both of these areas of the flower. When AB is present, as in *Pal*⁺ alleles, *del* only affects *pal* expression in the tubes, suggesting that there is a mechanism that masks the effect of *del* in the lobes. We propose that this masking is the result of the presence of lobe-specific factors. In the absence of an active *Del*⁺ product (*D*), there are presumably one or more factors (*L*) responsible for maintaining the wild-type levels of *Pal*⁺ expression in the lobes. If both *D* and *L* are transcription activators that interact with the same site, and *L* has a much higher affinity than *D* for the site or is more efficient at activating transcription than *D*, removal of *D* will have little or no effect on transcription (see Fig. 7). If deletion of AB affected the interaction of *pal* with *L* much more than with *D*, an effect of *D* on the

Figure 7. Model for the spatial patterns of expression of *Pal*⁺ and *pal-32*. The D factor is represented by red circles, and the L factor, by blue circles. Open ellipses represent factors interacting with regions AB, and yellow ellipses represent factors binding to the region proximal to the transcription initiation site (to the right of AB). Arrows indicate the levels of *pal* expression (for details, see text).

lobes would become apparent because D would no longer be efficiently masked by L. Such a model also explains why the AB deletion reduces transcription in the lobes more than in the tubes. According to this view, the AB region binds common rather than spatially specific transcription factors, and its effect on spatial pattern results from altered interactions between these common factors and spatially distributed factors such as L. A precedent for this type of model is that proposed for the regulation of the immunoglobulin genes (Staudt et al. 1986; Scheidereit et al. 1987). In this case, two transcription factors bind to the same octamer, but only one of the factors is bound in B lymphocytes, even though both factors are present. It has been suggested that other factors, which need not be tissue specific, interact differentially with the octamer-binding transcription factors and thus determine which interaction occurs.

Alleles lacking region ABC are uncoupled from regulation by *del*, suggesting that region C binds either D or a protein that interacts with D. Interestingly, region C contains a CAAT box, which is a sequence that has been shown to interact with families of diverse factors (Dorn et al. 1987). Therefore, this may be a candidate for the site of action of D and L, although other possibilities cannot be excluded because region C is 79 bp long. In the absence of regions ABC, *pal* expression is restricted to the base of the tube at ~1% of the wild-type level. Presumably, there are factors that activate *pal* expression at the base of the tube, which act in combination with factors bound in regions ABC to give wild-type levels of expression in this area.

An inversion that replaces ABC and all sequences farther upstream by a new sequence gives a very different pattern of expression from the other alleles. This allele

is also uncoupled from regulation by *del*, but its expression at the base of the tube is >20-fold greater than alleles lacking only region ABC and is also much higher than *Pal*⁺; *del* genotypes. Therefore, the pattern of expression cannot be explained simply by the loss of sequences involved in activation of transcription. Either the inversion removes transcription inhibitory sequences located >190 bp upstream or it introduces novel regulatory sequences (Coen et al. 1988; Robbins et al. 1989). In either case, this allele reveals a further spatial pattern of factors that can potentially interact with *pal*.

The results discussed above suggest that *Pal*⁺ expression results from an interaction of the upstream region with a prepattern of multiple common and spatially specific transcription factors in the flower. Studies in other systems indicate that multiple interactions between transcription factors in different areas or tissues are a general feature of eukaryotic genes. This suggests that most genes could potentially have multiple alleles with different patterns of expression analogous to the *pal* alleles, providing a very large source of genetic variation. How have such complex regulatory systems evolved? Two mechanisms could result in a gene coming under the control of a novel transcription factor. The binding specificity of the factor could change so that it recognized an upstream sequence of the gene. However, this would have pleiotropic consequences because it may uncouple the genes already regulated by the factor and it may also bring other genes under the control of the factor. A more likely mechanism, without these pleiotropic effects, is that the upstream region of the gene could mutate to a sequence recognized by the factor, hence, bringing the gene under its control. This suggests that changes in the upstream regions of genes may be the most common and flexible way for regulatory systems to evolve and may account for the current interaction of multiple factors with these regions. This is supported by the observation that interspecific differences in the regulation of the alcohol dehydrogenase (ADH) gene of *Drosophila* are the result of *cis*-acting mutations in the ADH gene (Dickinson and Carlson 1979; Brennan et al. 1988). The different patterns of expression of the *pal* alleles may therefore reflect the complex evolutionary history of the *pal* upstream region.

Materials and methods

Antirrhinum stocks

pal alleles have the same numbers as the lines in which they are maintained. *pal* lines JI501, JI520, JI518, JI33, JI32, JI15, and JI41 and the standard wild-type JI7 have been described previously (Fincham and Harrison 1967; Coen et al. 1986). Allele *pal-35G* was obtained from Gatersleben, courtesy of Dr. C. Lehmann. The line containing *pal-35G* was also mutant for *del*. Allele *pal-35G* was transferred into a *Del*⁺ background by crossing to JI 15. The *pal-569* allele was from a 'Kimosy white' line, kindly supplied by J.M. Pfister (Pfister and Widholm 1984; Martin et al. 1987). Kimosy white lacks any anthocyanin pigment because it also carries a *del* mutation. In a *Del*⁺ background, *pal-569* confers pigmentation restricted to a ring at the

base of the tube, similarly to *pal-15*. J18 carries a *del* allele in an otherwise wild-type background and shares a common origin with J17. Lines containing *pal-15*, *pal-32*, or *pal-41* in a *del* background were obtained from crosses between J15, J132, or J141 and the *del* line J18. Plants carrying *pal-33* in a *del* background were obtained by crossing J133 with another *del* line, J16, also wild type with respect to other genes of the anthocyanin pathway.

Molecular analysis

The methods for anthocyanin, RNA, and DNA analysis have been described previously (Coen et al. 1986; Robbins et al. 1989). The probes used in slot-blots and Northern blots were a 1.4-kb *EcoRI*–*HindIII* fragment of the *pal* clone pJAM4 (Coen et al. 1986), a 5.7-kb *EcoRI* fragment of the *niv* clone pAm3 (Wienand et al. 1982), and a cDNA clone of the *inc* gene (C. Martin, unpubl.). The blots were first probed with *pal* and then with either *niv* or *inc* after washing the *pal* signal with $0.1 \times$ SSC, 0.5% SDS at 70°C. The *pal* alleles were analyzed by Southern blotting using a 4.3-kb *EcoRI* fragment of the *pal* clone pJAM501 (Coen et al. 1986). For sequence analysis, each of the *pal* alleles was cloned into λ NM1149 (Murray 1982), as described previously (Coen et al. 1986). A *HindIII* fragment containing the site of excision of Tam3 was isolated from each of the clones, ligated into M13mp19 (Norranders et al. 1983), and sequenced by the dideoxy chain-termination method (Sanger et al. 1977).

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References

- Baur, E. 1924. Untersuchungen über das Wesen, die Entstehung und die Vererbung von Rassenunterschieden bei *Antirrhinum majus*. *Bibl. Genet.* 4: 1–70.
- Brennan, M.D., C.Y. Wu, and A.J. Berry. 1988. Tissue-specific differences for the alcohol dehydrogenase genes of Hawaiian *Drosophila* are conserved in *Drosophila melanogaster* transformants. *Proc. Natl. Acad. Sci.* 85: 6866–6869.
- Campuzano, S., L. Carramolino, C.V. Cabrera, M. Ruiz-Gomez, R. Villares, A. Boronat, and J. Modolell. 1985. Molecular genetics of the *achaete-scute* gene complex of *D. melanogaster*. *Cell* 40: 327–338.
- Carthew, R.W., L.A. Chodosh, and P.A. Sharp. 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. *Cell* 43: 439–448.
- Chen, S.M. and E.H. Coe, Jr. 1977. Control of anthocyanin synthesis by the *C* locus in maize. *Biochem. Genet.* 15: 333–346.
- Chia, W., G. Howes, M. Martin, Y. Meng, K. Moses, and S. Tsubota. 1986. Molecular analysis of the *yellow* locus of *Drosophila*. *EMBO J.* 5: 3597–3605.
- Coen, E.S. and R. Carpenter. 1986. Transposable elements in *Antirrhinum majus*: Generators of genetic diversity. *Trends Genet.* 20: 292–296.
- . 1988. A semi-dominant allele, *niv-525*, acts in *trans* to inhibit the expression of its wild-type homologue in *Antirrhinum majus*. *EMBO J.* 7: 877–883.
- Coen, E.S., R. Carpenter, and C. Martin. 1986. Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. *Cell* 47: 285–296.
- Coen, E.S., J. Almeida, T.P. Robbins, A. Hudson, and R. Carpenter. 1988. Molecular analysis of genes determining spatial patterns in *Antirrhinum majus*. In *Temporal and spatial regulation of plant genes* (ed. D.P.S. Verma and R.B. Goldberg), pp. 63–82. Springer-Verlag, Wien, New York.
- Coen, E.S., T.P. Robbins, J. Almeida, A. Hudson, and R. Carpenter. 1989. Consequences and mechanisms of transposition in *Antirrhinum majus*. In *Mobile DNA* (ed. D. Berg and M. Howe), pp. 411–434. American Society for Microbiology, Washington, D.C.
- Cone, K.C., F.A. Burr, and B. Burr. 1986. Molecular analysis of the maize anthocyanin regulatory locus *C1*. *Proc. Natl. Acad. Sci.* 83: 9631–9635.
- Davison, D., C.H. Chapman, C. Wedeen, and P.M. Bingham. 1985. Genetic and physiologic studies of a portion of the *white* locus participating in transcriptional regulation and in synapsis-dependent interactions in *Drosophila* adult tissues. *Genetics* 110: 479–494.
- Dean, C., J. Jones, M. Favreau, P. Dunsmuir, and J. Bedbrook. 1988. Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acids Res.* 16: 9267–9283.
- Dickinson, W.J. and H.L. Carlson. 1979. Regulation of the tissue specificity of an enzyme by a *cis*-acting genetic element: Evidence from interspecific *Drosophila* hybrids. *Proc. Natl. Acad. Sci.* 76: 4559–4562.
- Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis. 1987. A multiplicity of CCAAT box-binding proteins. *Cell* 50: 863–872.
- Fincham, J.R.S. and B. Harrison. 1967. Instability at the *Pal* locus in *Antirrhinum majus*. II. Multiple alleles produced by mutation of one original unstable allele. *Heredity* 22: 211–227.
- Fluhr, R., C. Kuhlmeier, and N.-H. Chua. 1986. Organ-specific and light-induced expression of plant genes. *Science* 232: 1106–1112.
- Forkmann, G. and G. Stotz. 1981. Genetic control of flavanone-3-hydroxylase and flavonoid-3'-hydroxylase activity in *Antirrhinum majus* (snapdragon). *Z. Naturforsch.* 36c: 411–416.
- Geyer, P.K., M.M. Green, and V.C. Corces. 1988. Reversion of a gypsy-induced mutation at the *yellow* locus of *Drosophila melanogaster* is associated with the insertion of a newly defined transposable element. *Proc. Natl. Acad. Sci.* 85: 3928–3942.
- Giuliano, G., E. Pichersky, V.S. Malik, M.P. Timko, P.A. Scolnik, and A.R. Cashmore. 1988. An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci.* 85: 7089–7093.
- Heller, W., G. Forkmann, L. Britsch, and H. Grisebach. 1985. Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-cis-diols with flower extracts from *Matthiola incana* and its role in anthocyanin biosynthesis. *Planta* 165: 284–287.
- Kristiansen, K.N. 1984. Biosynthesis of proanthocyanidins in barley: Genetic control of the conversion of dihydroquercetin to catechin and procyanidins. *Carlsberg Res. Commun.* 49: 503–524.
- Martin, C.R., R. Carpenter, E.S. Coen, and A.G.M. Gerats. 1986. The control of floral pigmentation in *Antirrhinum majus*. *Soc. Exp. Biol. Symp.* 32: 19–52.
- Martin, C.R., R. Carpenter, H. Sommer, H. Saedler, and E.S. Coen. 1985. Molecular analysis of instability in flower pigmentation in *Antirrhinum majus*, following isolation of the

- pallida* locus by transposon tagging. *EMBO J.* **4**: 1625–1630.
- Martin, C., E. Coen, T.P. Robbins, J. Bartlett, J. Almeida, and R. Carpenter. 1987. The control of floral pigmentation in *Antirrhinum*. *Biochem. Soc. Trans.* **15**: 14–17.
- Murray, N.E. 1982. Phage lambda and molecular cloning. In *Lambda II* (ed. R.W. Hendrix, J.W. Roberts, F.W. Stahl, and R.A. Weissberg), pp. 395–442. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Norlander, J., T. Kempe, and J. Messing. 1983. Construction of improved vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**: 101–106.
- Paz-Ares, J., D. Ghosal, U. Wienand, P.A. Peterson, and H. Saedler. 1987. The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. *EMBO J.* **6**: 3553–3558.
- Pfister, J.M. and J.M. Widholm. 1984. Plant regeneration from snapdragon tissue cultures. *Hortscience* **19**(6): 852–854.
- Pirrotta, V., H. Steller, and M.P. Bozzetti. 1985. Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. *EMBO J.* **4**: 3501–3508.
- Robbins, T.P., R. Carpenter, and E.S. Coen. 1989. A chromosome rearrangement suggests that donor and recipient sites are associated during Tam3 transposition in *Antirrhinum majus*. *EMBO J.* **8**: 5–13.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Scheidereit, C., A. Heguy, and R.G. Roeder. 1987. Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter *in vitro*. *Cell* **51**: 783–793.
- Schulze-Lefert, P., J. Dangel, M. Becker-Andre, K. Hahlbrock, and W. Schulz. 1989. Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J.* **8**: 651–656.
- Schwarz-Sommer, Z., N. Shepherd, E. Tacke, A. Gierl, W. Rohde, L. Leclercq, M. Mattes, R. Berndtgen, P.A. Peterson, and H. Saedler. 1987. Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. *EMBO J.* **6**: 287–294.
- SivaRaman, L. and B. Thimmappaya. 1987. Two promoter-specific host factors interact with adjacent sequences in an EIA-inducible adenovirus promoter. *Proc. Natl. Acad. Sci.* **84**: 6112–6116.
- Staudt, L.M., H. Singh, R. Sen, T. Wirth, P.A. Sharp, and D. Baltimore. 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. *Nature* **323**: 640–643.
- Stern, C. 1954. Two or three bristles. *Am. Sci.* **42**: 213–247.
- . 1968. *Genetic mosaics and other essays*, Harvard University Press, Cambridge.
- Stickland, R.G. and B.J. Harrison. 1974. Precursors and genetic control of pigmentation. I. Induced biosynthesis of pelargonidin, cyanidin, and delphinidin in *Antirrhinum majus*. *Heredity* **33**: 108–112.
- Wheldale, M. 1907. The inheritance of flower colour in *Antirrhinum majus*. *Proc. Roy. Soc. London Ser. B.* **79**: 288–305.
- Wienand, U., H. Sommer, Z. Schwarz, N. Shepherd, H. Saedler, F. Kreutzaler, H. Ragg, E. Fautz, K. Hahlbrock, B.J. Harrison, and P.A. Peterson. 1982. A general method to identify plant structural genes among genomic DNA clones using transposable element induced mutations. *Mol. Gen. Genet.* **187**: 195–201.
- Willmitzer, L. 1988. The use of transgenic plants to study plant gene expression. *Trends Genet.* **4**: 13–18.
- Yu, Y.T. and J.L. Manley. 1984. Generation and functional analyses for base-substitution mutants of the adenovirus 2 major late promoter. *Nucleic Acids Res.* **12**: 9309–9321.

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