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.

[Key Words: Allelic series; gene regulation; transposons; plants]

Received June 16, 1989; revised version accepted August 22, 1989.

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^noted alleles in Drosophila, which change the distribution of pigment between testis and eye and uncouple white from inhibition by the zeste^1 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^recessive], 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...
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](https://genesdev.cshlp.org/1759.png)

**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.
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Figure 1 shows the pattern of pigmentation in the corolla of flowers carrying the alleles indicated. The quantity of \textit{pal} mRNA at the base of the tube of \textit{pal-15} was reduced to \(~1\%\) of that in the \textit{pal-520} control in the same area (Fig. 2c). No transcript was detected in the remainder of the corolla for \textit{pal-15}, whereas higher levels of \textit{pal} mRNA were found in the rest of the corolla than at the base of the tube in \textit{pal-520} (Fig. 2c). These results show that distinct patterns of color conferred by the \textit{pal} alleles reflect different spatial distributions of \textit{pal} mRNA. As a control, the same blots were probed with a clone of the \textit{nivea} (\textit{niv}) gene that encodes chalcone synthase, an enzyme also required for anthocyanin synthesis (Wienand et al. 1982). The levels and spatial distributions of \textit{niv} transcript were similar, irrespective of \textit{pal} genotype (Table 1; Fig. 2).

In another control, the relative levels of \textit{pal} and \textit{niv} transcripts produced in the upper and lower halves of the corolla in flowers from the lines containing \textit{pal-501} or \textit{pal-518} were determined. Transcripts of \textit{pal} and \textit{niv} were found to be distributed evenly between the two areas in both lines (Fig. 2d). Transcription initiation was also investigated to determine whether \textit{pal} mRNA detected in blots was accurately initiated. S1 mapping of RNA produced by \textit{pal-501}, \textit{pal-518}, \textit{pal-33}, \textit{pal-32}, and \textit{pal-15} revealed that the sites of transcription initiation in all of these alleles were identical to those found previously in \textit{Pal} \(^+\) and other mutant alleles (Coen et al. 1986; Robbins et al. 1989; data not shown).

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

| Allele | \(T\) | \(L\) | \(T/L\) | \(T\) | \(L\) | \(T/L\) |
|--------|------|------|--------|------|------|--------|
| \textit{pal} | \textit{mRNA} | \textit{niv} |
| 501 | 100 | 440 | 0.25 | 100 | 200 | 0.5 |
| 520 | 110 | 470 | 0.25 | 130 | 190 | 0.7 |
| 518 | 100 | 130 | 0.80 | 25 | 20 | 1.2 |
| 33 | 60 | 50 | 1.20 | 25 | 10 | 2.5 |
| 32 | 10 | 5 | 2.00 | 4 | 1 | 4.0 |

All measurements are relative to the levels detected in the tubes of \textit{pal-501}, which confers wild-type phenotype. \(T/L\) is the ratio of the levels in tubes and lobes. Alleles \textit{pal-501}, \textit{pal-520}, and \textit{pal-518} are maintained in isogenic backgrounds because they were derived by selfing a common progenitor containing \textit{pal-2}, which has been inbred for many generations. Allele \textit{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 levels of pigmentation in the lobes is much more reduced than in the tubes. Alleles \textit{pal-33} and \textit{pal-32} were selected in progenies of crosses between lines containing \textit{pal-2} and other lines carrying mutant \textit{pal} alleles. The S.E.M., based on five independent measurements, is 10–20% for the anthocyanin levels shown. The levels of \textit{pal} and \textit{niv} mRNAs were determined by slot-blot analysis.
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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 muta-
tants, 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, genes in the lobes were unaffected by (Fig. 4). How-
ever, the quantity of del or Del* genes in the tubes of pal was reduced ~100-

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 ex-

tacted 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.

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.

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-
teion 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.

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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.
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-1. 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].
Genetic interactions underlying color patterns

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 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.

sistant 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.

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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].

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We have shown that different patterns of expression in 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; Cartewh 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 EIIA-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 prepatter 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 ci 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
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, which act in combination with factors bound in regions ABC to give wild-type levels of expression in this area.
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 J115, 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-blotting 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 x 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 [Norlander et al. 1983], and sequenced by the dideoxy chain-termination method [Sanger et al. 1977].

Acknowledgments

We thank John Firmin for synthesizing leucocyanidin, Hans Sommer for providing pAM3, Andrew Davies and Peter Scott for photography, and David Hopwood and Caroline Dean for constructive comments on the manuscript.

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*Genes Dev.* 1989, 3:
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