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Evidence for Correlation Between Mutability of an Unstable Anthocyanin-governing Locus and Abundance of Anthocyanin

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Summary. The *pal-rec* gene of *Antirrhinum majus* suppresses anthocyanin except in those cell lines where *pal-rec* has mutated to *Pal,* so that anthocyanin-coloured flecks appear on whitish petals. *Antirrhinum majus* families of very high and very low anthocyanin content (Dark and Pale) were obtained and crossed with two *pal-rec pal-rec* lines, one with consistently high and the other consistently low mutability. Mutable offspring from Dark parents tended to show higher mutability than those from Pale parents in crosses with either mutable line, providing evidence for an association between intense pigmentation and high mutability. Such an association is discussed in the context of relationship between precursor availability for conversion by a gene product and initiation of activity of that gene.

Key words: Mutability – Regulation – Anthocyanin content – *Antirrhinum majus*

1 Introduction

The *pallida* gene of *Antirrhinum majus* governs the synthetic step from flavanonal to anthocyanin (Harborne 1967; Grisebach 1972). The unstable allele *pal-rec* while in its initial recessive condition abolishes anthocyanin, but mutates to *Pal,* each such event giving a pigmented spot. Fewer than 100 spots to more than 10,000 may appear on each large corolla lobe (see Jeffries 1977; Jeffries and Sastry 1981). Influences on mutation frequency include temperature (Harrison and Fincham 1964), genetic constitution at modifying loci, e.g. *Stabiliser* (Harrison and Fincham 1968), and a two-part controlling system that confers instability of the mutation rate itself (Sastry et al. 1980; Jeffries and Sastry 1981). In addition to these influences, loci governing steps in the flavonoid pathway prior to *pallida* may exert an effect. *Eosinea* governs the second hydroxylation on the B ring of the C15 flavonoid skeleton. Pink *eos eos* flowers contain less of their pelargonin than *Eos*/-flowers do of their wildtype magenta cyanin (Jorgensen and Geissman 1955) and *eos eos pal-rec pal-rec* flowers have far fewer *Pal* spots on average than comparable *Eos*/- *pal-rec pal-rec* flowers (Harrison and Fincham 1963; Jeffries and Sastry 1981). *Incolorata* mediates the flavanone to flavanonol step, *inc inc* plants therefore lacking anthocyanin, and *Inc inc* plants are paler than *Inc Inc* sibs (Sampson and Hunter 1959). *Inc inc pal-rec*/- plants mutate merely later than their *Inc Inc* counterparts somatically, but germinally they mutate far less frequently (see Fincham 1973). Rare late somatic mutation in *inc inc pal-rec pal-rec* flowers has been demonstrated by in vitro feeding of dihydroquercetin to buds (Harrison and Stickland 1974).

The possibility therefore arises that the product of dominant genes preceding *pal* in the flavonoid pathway are necessary for maximum mutability of *pal-rec,* and indeed Harrison and Carpenter (1973) obtained direct evidence for such a relationship in the case of *nivea,* which governs C15 formation. Fincham (1973) suggested that it is accumulation of precursor for the *Pal* gene product which triggers maximum mutability of *pal-rec* in the same way that it might switch on activity of *Pal* in the wildtype stable dominant condition, both actions occurring at the developmental stage appropriate to anthocyanin synthesis.

The present study examines mutability of *pal-rec* in lines of relatively strong and relatively weak anthocyanin-producing potential. In such plants, wildtype for anthocyanin production except for *pal-rec,* it has been possible to demonstrate higher average mutability in dark than in pale backgrounds, providing further support for the hypothesis that precursor availability influences mutation rate to the dominant allele.

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2 Materials and Methods

2.1 Production of Dark and Pale lines

Eight commercial brands of *Antirrhinum majus* were grown outdoors, and *Niv/- Inc/- Pal/-* plants compared visually for colour intensity. (More detailed genotypes and description appear in Jeffries (1977)). Seven very dark and five very pale individuals were selected, of which ten were clearly *eos eos;* colour depth was easier to assess in the pink *eos eos* flowers. All twelve selected plants were brought into the greenhouse and crossed to an inbred line with entirely pink flowers of medium anthocyanin intensity, *Niv Niv, Inc Inc Pal Pal eos eos.* All other plants used were pot grown in the greenhouse.

The offspring showed one of the commercial pale parents to be *pal-car pal-car,* an allele allowing for about half the anthocyanin production of *Pal Pal* (see Fincham and Harrison 1967), and it was discarded. In the other offspring families dark and pale pigment intensities were expressed comparably to that of the commercial parents, indicating that at least in the genetic background of this tester line the inferred modifiers from the commercial parents are not recessive. Semidominant or dominant modifiers were required for the genetic programme.

Each of the seven dark and four remaining pale commercial plants was crossed to another inbred tester line, homozygous for *pal-tub,* which permits only a ring of anthocyanin at the base of the corolla tube in *Eos/-* flowers, and none in *eos eos.* This tester line, *Niv Niv Inc Inc pal-tub pal-tub Eos Eos* gives visually medium anthocyanin intensity in crosses to the *pal-rec pal-rec* lines maintained at Leeds, to which it is closely related. Ten offspring were raised from each cross to this line, and as in the previously described crosses, the inferred colour modifiers proved non-recessive. Darkest or palest individuals were selected from each family and the breeding scheme continued as set out in Table 1. It aimed to conserve all non-recessive modifiers that affect anthocyanin content only quantitatively, except for intermediate alleles of *pallida* (see Fincham and Harrison 1967). It aimed to eliminate any recessive alleles on the flavonoid pathway or genes altering flavonoid distribution carried by the commercial plants (see Stubbe 1966); and any direct influences on mutability without other known effect on pigmentation, such as *Stabiliser.*

At each "Stage" in the programme enough plants were raised to allow for the possibility of up to three "*D*" modifiers segregating. It was possible to be reasonably confident of seeing at least one plant in each family displaying a visually additive effect of *D1/- D2/- D3/-*. At Stages I, III, and IV each family was distributed by eye into subclasses according to the differences in depth of flower colour, and numbers of subclasses supported the assumption of more than one *D* modifier. All but members of the most extreme classes were discarded, except in Stage II where mostly *pal-tub pal-tub* segregants were raised.

The Pale lines were named Lines a, b, c, (and Z, the *pal-car pal-car*). The Dark lines were Lines d, e, f, g, h, x, and y. Line Z was discarded before any crosses were made to mutable lines, and the least extreme Dark lines x, and y, were discarded after Stage III. The eight lines remaining in Stage IV were more alike in flower size and shape and growth rate than they had been in Stages I and II.

Table 1. Breeding programme for Pale and Dark plants conserving inferred "D" loci which enhance or depress pigmentation

Two "*D*" loci are assumed since segregation of subclasses concerning pigment intensity indicated more than one *D* in each selected Dark and Pale line: see Materials and Methods. Only genotypes selected for crossing with High- and Low-mutable lines are shown at each Stage			Chance of visually selected plants bearing D_1 and D_2 [a]
Outcross		*Pal Pal eos eos* $D_1/-D_2/-$ [b]	
	From commercial seed		
		× *pal-tub pal-tub Eos Eos* $d_1\ d_1\ d_2\ d_2$	100%
		Inbred tester	
Stage I		*Pal pal-tub Eos eos* $D_1\ d_1\ D_2\ d_2$	100%
		Selfed	
		pal-tub/- eos/- [c]	56%
		Backcrossed to tester	
Stage III		*Pal pal-tub Eos/-* $D_1\ d_1\ D_2\ d_2$	
		Selfed	
Stage IV	(i)	*Pal pal-tub Eos/-* $D_1\ D_1\ D_2\ D_2$ (majority)	100%
	(ii)	*pal-tub pal-tub* genotypes (used to ensure plenty of *pal-rec pal-tub* offspring in crosses to *pal-rec pal-rec* lines since mutability is obscured in *Pal pal-rec* plants)	56%

[a] Assumes D_1 and D_2 are additive, semidominant and unlinked to *Pal*

[b] All chosen commercial plants were *eos eos* except one *Eos Eos* Dark plant. "*D*" loci are hypothetical non-recessive modifiers conferring Dark or Pale anthocyanin pigmentation

[c] Selected primarily for a separate study, see Jeffries 1977

2.2 Measurement of Pigment Intensity

The colour of *Antirrhinum majus* flowers of dominant genotype is determined mainly by their content of anthocyanin, cyanidin-3-rutinoside in *Eos/-* plants. Slight variation in degree of blueness depends on cell pH and metal chelation (Harborne 1967) and on degree of co-pigmentation with flavones (Asen et al. 1972). Environmental conditions were the same for all families. So plants selected by eye as darkest or palest were those whose genotypes most favoured and most restricted anthocyanin production in the flowers. Very deeply pigmented flowers could have contained some cyanidin-3-monoglucoside as well as the usual 3-rutinoside (Gilbert 1971), but this would not alter colour or intensity (Harborne 1967). Stage III plants were assessed only visually but in Stages I and IV spectral scans were obtained with petal extracts. Young flowers from the palest and darkest plants were frozen and thawed then chopped in MeOH plus 1% HCL, with which they were extracted twice by shaking at room temperature for 18 hours, then 6 hours. After removal of the bleached petal fragments by centrifugation, the bulked extracts were diluted to a standard volume and scanned in the visible spectrum. Pure cyanidin-3-rutinsode peaks at 523 mμ (Harborne 1967) but without prior renewal of co-pigments a higher wavelength is expected and the observed peaks fell between 536 mμ and 540 mμ. Peak height at standard dilution gave a "Colour Measure" per unit of tissue for the representatives of each visually selected line. These figures, together with parallel visual assessments, are given in Table 2. Agreement is mainly good between visual and spectrophotometric assessments. Colour Measures can be compared only within Stages as these flowered at different times of year (Stage I: May, II: November, IV: July) and had therefore experienced different growing conditions as the greenhouse used is not temperature-controlled.

2.3 Dark and Pale Lines Crossed with Mutable Lines

Mutability level of *pal-rec* is here defined by the number of mutational events on the face of one large corolla lobe. A visual scale was devised, ranging from class 0.0 (no *Pal/-* spots) to 8.0 (entirely *Pal/-*). Classes 0.5 to 4.0 revealed 100–8000 spots per lobe when precise counts were made with the aid of a microscope. In classes above 4.0 individual events are obscured by confluence, but it is estimated that class 7.0 represents more than 10^4 mutations per lobe.

Members of high-mutable and low-mutable families of Leeds stock *pal-rec pal-rec Eos Eos* plants were crossed to each selected Dark and Pale plant. The high- and low-mutable lines had been derived by selection from mutable families whose mutability level ranged e3er five classes as is typical of non-inbred families (see Jeffries and Sastry 1981). The resulting high-mutable line fell within classes 5–6, and the low-mutable line 1–2, the relative infrequency of *Pal/-* spots being independent of the *Stabiliser* locus since the line was free of its mutability-depressing allele *St.*

At each Stage the selected members of Dark and Pale families were all crossed to both a high-mutable and a low-mutable family. In the most frequent configuration (see Table 1) the Dark or Pale plant was visually selected for possession of the inferred non-recessive *D* alleles which enhance or depress anthocyanin synthesis. If the selected phenotype resulted from two such *D* alleles the cross would be: –

$$\frac{Pal\ pal\text{-}tub\ Eos/\text{-}\ D_1\ d_1\ D_2\ d_2}{\text{Pale or Dark}} \times \frac{pal\text{-}rec\ pal\text{-}rec\ Eos\ Eos\ d_1\ d_1\ d_2\ d_2}{\text{High- or Low-mutable}}$$

(*Pal pal-rec* segregants discarded)

pal-tub pal-rec Eos/-D_1/-D_2/-	25%
pal-tub pal-rec Eos/-d_1d_1 D_2/-	25%
pal-tub pal-rec Eos/-D_1/-d_2d_2	25%
pal-tub pal-rec Eos/-d_1d_1 d_2d_2	25%

Table 2. Pigment intensity of Dark and Pale lines

	Stage I			Stage III	Stage IV		
	Colour measure[a]	Order of colour intensity		Order of colour intensity by eye	Colour measure	Order of colour intensity	
		(i) by colour measure	(ii) by eye			(i) by colour measure	(ii) by eye
Pale lines							
a	2.15	3	2	1	0.47	1	1
b	2.05	2	2	2	1.11	3	3
c	3.40	4	2	2	0.94	2	2
z	0.79	1	1				
(*pal-car pal-car*)							
Dark lines							
d	9.45	2	1	1	5.95	2	1
e	8.90	4	2	2	5.78	3	2
f	–	?[b]	2	3	6.60	1	3
g	9.60	1	2	3	5.18	4	1
h	9.20	3	2	3	5.07	5	4
x	5.90	5	4	4			
y	5.00	6	3	5			

[a] Maximum peak within 536–540 mμ obtained for solution of extracted flavonoids per unit of tissue at standard dilution. Solutions obtained from samples of 15, 20, or 30 young flowers

[b] Not measured

The proportion of the mutable offspring that carries all of the colour modifiers from the Pale or Dark parent would be only 13.5% if 3 *D* alleles were involved. It was sometimes apparent from the colour intensity of its *Pal*/- spots whether an offspring plant was strongly colour modified, but assessment of such small pigmented areas was not precise enough to allow classification of offspring families into subclasses according to constitution at the inferred *D* loci. All mutable offspring were therefore scored, and a single average score obtained for each family to compare with parallel families. Scores were obtained by visual allocation to mutability classes of inflorescences bearing eight or more flowers.

3 Results and Discussion

Average scores of Dark and Pale mutable families fall into 21 Sets, each of which represents a scoring date, since precisely controlled environment facilities were not available. Temperature is known to affect mutability level (Harrison and Fincham 1964), so scores can only be validly compared if all inflorescences have experienced similar temperature fluctuations.

Table 3 presents differences in average scores for Dark and Pale mutable families in each of the 21 Sets, together with the number of individual scores contributing to each pair of compared averages. The totals for the columns summarise how these results bear on the hypothesis of mutability promotion in a deeply pigmented background. Supporting evidence exceeds the contradictory.

Table 4 shows the relative contributions of each Stage. They are enumerated by multiplying each Dark – minus – Pale mutability difference listed in Table 3 by its number of contributory inflorescence scores. The resulting empirical figures are separately summed for each Stage into a positive and a negative total which then represent the weight of supporting and contradictory evidence respectively for the hypothesis that greater pigment intensity tends to accompany higher

Table 3. Comparisons of average mutability scores for offspring of Dark and Pale plants crossed to uniformly mutable lines

Stage	Set	Crosses to high-mutable line		Crosses to low-mutable line	
		Mutability score by which Dark families on average exceed Pale families	Number of scores involved	Mutability score by which Dark families on average exceed Pale families	Number of scores involved
I	1	+0.47	44	−0.11	24
	2	+1.05	27	+0.73	17
	3	−0.28	25	+0.68	18
II	10	+1.33	39	+0.15	40
	11	+0.94	44	+0.16	42
	12	−0.15	33	+0.08	30
	13	+0.30	9	−0.20	16
	14	+0.41	11	−0.15	29
	15	+0.06	45	−0.02	70
	16	−0.16	61	+0.03	84
	21	−0.61	7		
III	4	+0.97	25	−0.26	20
	5	+1.22	37	−0.06	13
	6	+0.74	40		
	7	+1.41	38		
	8	+0.73	16		
	9	+1.84	23		
IV	17	+1.61	66	−0.42	165
	18	−1.35	30	−1.04	37
	19	+0.50	356	+0.64	480
	20	−1.19	58		
Totals		15 Comparisons: +	820	7 Comparisons: +	711
		6 Comparisons: −	214	8 Comparisons: −	374

Overall totals
22 Comparisons: + (1531 Scores)
14 Comparisons: − (588 Scores)

Table 4. Ratio of negative to positive evidence for an association between high mutability and intense pigmentation

Stage	(i) High-mutable parent		(ii) Low-mutable parent		(i) plus (ii)		Ratio of neg : pos
	Positive total[a]	Negative total	Positive total	Negative total	Positive total	Negative total	
I	49.03	7.00	24.65	2.65	73.68	9.64	1 : 7.64
II	103.14	18.98	17.64	8.95	120.78	27.93	1 : 4.32
III	206.57	0.00	0.00	5.98	206.57	5.98	1 : 34.54
IV	323.44	69.02	307.20	104.48	630.64	173.50	1 : 3.63
I – IV combined	682.18	95.00	349.49	122.05	1031.67	217.05	
Ratio of neg : pos	1 : 7.18		1 : 2.86		1 : 4.75		

[a] "Positive" and "Negative" totals obtained from: – (mutability scale units by which average score for Dark families exceeds that for Pale families) multiplied by (number of inflorescence scores involved)

mutability. All Stages support the hypothesis but to degrees which do not reflect expectations from genotypes at the inferred *D* loci (see Table 1). Stage IV provides the highest proportion in each line of visually selected plants probably homozygous for all its colour modifiers, but does not provide the greatest support for the hypothesis. In other Stages the majority of mutable offsprings which provide the data must be homozygous for the recessive counterparts of at least some of the inferred colour modifying alleles, due to the coloured parents being heterozygous at all *D* loci. This necessary departure from a maximally colour-modified genotype in the case of most plants crossed to the High- and Lowmutable lines renders the proposed association more persuasive. Sets of offspring most often give higher average scores if the parent is Dark-modified than if the parent is Pale-modified, although most *Pal* parents are only partialy colour-modified. It cannot be ruled out that one or more Dark or Pale lines could have been represented solely by plants bearing *St*, although no line contributed markedly more or less than the others to the overall trend when traced through successive stages. Nor can it be ruled out that minor or recessive modifiers of mutability or colour intensity went undetected by the programme of Table 1. When rendered homozygous these could interfere with expression of a relationship between pigment intensity and mutability level. Nevertheless, such a relationship was demonstrated. The overall trend thus supports the existence of a correlation between colour intensity and mutability level sufficiently to merit further investigation.

The possibility of such a relationship remains to be investigated in other plants, the only comparable evidence being that of Harrison and Carpenter (1973) on doubly mutable *Antirrhinum*. They treated niv-rec niv-rec pal-rec pal-rec flowers to reveal flavonoid-containing *Niv* areas in relation to anthocyanin-coloured *Niv Pal* doubly mutated areas and found that mutation *niv-rec* → *Niv* preceded *pal-rec* → *Pal* more often than by chance especially in early flower development, suggesting a functional relationship between precursor presence and mutation to the functional gene.

It is not certain that anthocyanin content of mature flowers as here measured reflects precursor concentration available to influence *pal-rec* during differentiation of flower tissue, and the tendency demonstrated, if indicating any functional relationship, may indicate a more indirect one than here proposed. Nevertheless, if mature anthocyanin content was a fair reflection of previous precursor availability it would be reasonable to suggest on the present evidence the promotion of mutability by precursor accumulation.

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