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Genes affecting flower colour and pH of flower limb homogenates in *Petunia hybrida*

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Summary. In *Petunia hybrida* four complementary genes are present, each having, if homozygous recessive a blueing effect on the flower colour. These genes have no qualitative or quantitative effect on anthocyanins and flavonols. In mutants homozygous recessive for one (or more) of the *Ph* genes the pH of aqueous flower limb homogenates is increased. It is assumed that the *Ph* genes in *Petunia* are involved in maintaining the pH in the vacuole fluid in the flower.

Key words: *Petunia hybrida* – Flower colour genes – pH flower limb homogenates

Introduction

The colour of flowers is influenced by the hydroxylation and glycosidation patterns of anthocyanins as well as by the complex forming of anthocyanins with metal ions and the copigmentation with other pigments. An influence of the pH of the cell sap has also been suggested (Stewart et al. 1975).

Although direct measurement of the pH in flower epidermis cells is not possible, indirect methods (Stewart et al. 1975) on very small samples from specific flower tissue areas, provide strong evidence that the pH of the cell sap has an influence on the flower colour of flowers coloured by anthocyanins: the flower colour is more bluish if the pH is higher.

Preliminary experiments with *Petunia hybrida* have shown that the most bluish flower types contain petunidin or malvidin-3 rutinoside (p-coumaryl)-5 glucoside, are co-pigmented by flavonol and have an aqueous flower homogenate pH of about 6.0. More reddish flower types containing the same amount of the same anthocyanin and flavonol have a pH of about 5.5. Aqueous leave homogenates have a pH of about 5.9 independent of the flower colour. As far as we know no monofactorial genetic differences for the pH of flower homogenates have been described in literature.

In *Petunia hybrida* five genes have been identified (*Ph1, Ph2, Ph3, Ph4:* Wiering et al. 1979; *Ph5:* Vallade and Cornu 1979). Each, if homozygous recessive, has a blueing effect on the flower colour.

Crossed with each other, lines homozygous recessive for different *Ph* genes, show genetic complementation.

In this paper we show that there is a strong correlation between flower colour, pH of aqueous flower limb homogenates and genotype for four of the above mentioned genes.

Materials and methods

Phenotypes and genotypes

Data of the lines used, the F1's and non parental types segregating in the crosses are given in Table 1.

The genes described in this paper are as follows:

Hf1 – : 3′-5′ hydroxylated anthocyanins (delphinidin, petunidin or malvidin) present

hf1hf1: only 3′ hydroxylated anthocyanins (cyanidin or peonidin) present

Hf2 – : 3′-5′ hydroxylated and 3′ hydroxylated anthocyanins present (incomplete hydroxylation); there is no effect in the flower tube

hf2hf2: only 3′ hydroxylated anthocyanins present

Rt – : 3 rhamnosylated anthocyanins (cyanidin-3 rutinoside or delphinidin-3 rutinoside) present

rtrt: cyanidin-3 glucoside or delphinidin-3 glucoside present

Fl – : high concentration of flavonol (kaempferol or quercetine glycosides)

Table 1. Phenotype and genotype of lines, F1's and non parental types segregating in the crosses; genetical background *GfGf MtMt*

Line code	Phenotype					Genotype[b]							
	Flower colour	HCC[a]	Anthocyanin[c]	Flavonol	pH flower homogenate	*An1*	*Ph1*	*Ph2*	*Ph3*	*Ph4*	*Hf1*	*Rt*	*Fl*
Parental lines													
M1	magenta reddish	627	peonidin-3RGac5G	traces	5.48	+	+	+	+	+	–	+	–
V12	purple reddish	730/1	malvidin-3RGac5G	traces	5.50	+	+	+	+	+	+	+	–
R57	red 1 purplish	826/3	cyanidin-3G	traces	5.61	+	+	+	–	+	–	–	–
R62	red 1 purplish	824/3	cyanidin-3G	traces	5.57	+	+	+	+	–	–	–	–
M40	magenta purplish	32/2	peonidin-3RGac5G	traces	6.06	+	+	+	–	+	–	+	–
M51	magenta purplish	629	peonidin-3RGac5G	traces	5.90	+	+	–	+	+	–	+	–
V36	purple purplish	733	petunidin-3RGac5G	traces	6.10	+	–	–	+	+	+	+	–
V18	purple bluish	38	malvidin-3RGac5G	much	6.22	+	–	+	+	+	+	+	+
V28	purple bluish	937/1	petunidin-3RGac5G	much	5.91	+	+	–	+	+	+	+	+
W5	white		–	traces	5.90	–	+	+	+	+	–	+	–
F1's													
V18 × M1	purple middle	35/1	malvidin-3RGac5G	much		+	±	+	+	+	±	+	±
V28 × M1	purple middle	34 – 35	malvidin-3RGac5G	much		+	+	±	+	+	±	+	±
V12 × M40	purple reddish	30 – 30/1	malvidin-3RGac5G	traces		+	+	+	±	+	±	+	–
V12 × R62	purple reddish	730	malvidin-3RGac5G	traces		+	+	+	+	±	±	±	–
R62 × W5	magenta reddish	27/1	peonidin-3RGac5G	traces		±	+	+	+	±	–	±	–
Non parental types													
	red 1 reddish	621 – 622	cyanidin-3G	traces		+	+	+	+	+	–	–	–
	grey 1 reddish	0023	delphinidin-3G	traces		+	+	+	+	+	+/±	–	–
	grey 1 purplish	033/2	delphinidin-3G	traces		+	–	+	+	+	+/±	–	–
	magenta middle }	633/2 –	peonidin-3RG(ac)5G	much		+	+/± or	+/±	+	+	–	+/±	+/±
	magenta bluish }	640/2	peonidin-3RG(ac)5G	much		+	+	–	+	+	–	+/±	+/±

[a] Horticultural colour chart of the British colour council
[b] + = homozygous dominant; – = homozygous recessive; ± = heterozygous
[c] *G* = glucose; *R* = rhamnose; *ac* = p-coumaric acid

flfl: only traces of flavonol present
Gf–: 5 glucosylated 3 acylated anthocyanins present
gfgf: cyanidin-3 rutinoside or delpinidin-3 rutinoside present
Mt–: 3′ methylated anthocyanins present (peonidin, petunidin or malvidin)
An1–: coloured flowers
an1an1: white flowers
Ph–: all *Ph* genes dominant: flower colour of reddish type
phph: with at least one *Ph* gene homozygous recessive: flower colour of purplish type.

The genes *Ph1* and *Ph2* only affect flower colour. Of these genes, *Ph1* is strongly linked to *Hf1* and *Ph2* is situated on chromosome IV (Maizonnier and Moessner 1979).

The gene *Ph3* has some pleiotropic effects: plants homozygous recessive for this gene are female sterile and the expression of the dominant allele of *Hf2* is impossible (Wiering 1974). Lines homozygous recessive for *Ph3* are maintained by cuttings. *Ph3* is linked to the gene *An4* on chromosome VII.

The gene *Ph4* also has some pleiotropic effects: in *ph4ph4* plants also the dominant allele of *Hf2* does not come to expression. The gene is instable: reversion or mutation of *ph4* to *Ph4* results in reddish spots on a purplish background. *Ph4* is linked with *Ht1* on chromosome III.

The dominant allele of the gene for flower colour fading, *Fa,* is only expressed in *ph3ph3* and *ph4ph4* plants (de Vlaming et al. 1982).

pH measurement

Preliminary experiments have shown that the pH measurements have to be done with fresh flowers: withered flowers give a higher pH. Three fresh flower limbs of 1–3 day old mature flowers are, within 15 min after picking from the plant, washed twice with deionized water and then homogenized in 4 ml deionized water for 1 min in a Bühler homogenizing apparatus. Immediately after homogenizing, the pH is measured with a pH meter with a combined electrode (Radiometer pH 28). Every plant is measured twice, on different days. The mean values obtained are used in Fig. 1.

Standard deviations of about 20 measurements of cloned plants of the lines M1, R57, R62 and M40 are between 0.07 and 0.13 pH units.

Buffered 4M NaCl solutions

1/15 M Na_2HPO_4 and 1/15 M KH_2PO_4 solutions in 4M NaCl were mixed to obtain pH's in the range 4.6–6.5. One flower limb in 5 ml buffered NaCl solution is homogenized for 1 min. After centrifuging at 10,000 g/min for 10 min, the absorption spectrum of the supernatant is measured in a Zeiss DMR21 spectrophotometer.

The P values in the Tables are at the χ^2 level.

Results

In Tables 2–6 the segregations for flower colour controlled by the *Ph* genes are presented. Figure 1 shows the distribution of the pH values of flower homogenates of plants dominant or homozygous recessive for a *Ph* gene.

The gene Ph1

The segregation found in the cross V18 × M1 is presented in Table 2. Reddish and middle types appear to be dominant for the gene *Ph1,* bluish and purplish types homozygous recessive. The strong linkage found between the genes *Ph1* and *Hf1* corresponds with the results found in unpublished earlier experiments (0.4% cross-over).

Table 2. F2 and B1 of the cross V18 × M1; F1 $\frac{Ph1\ Hf1\ Fl}{Ph1\ hf1\ fl}$; B1 : F1 × V36; genetical background *RtRtPh2Ph2Ph3Ph3Ph4Ph4*

Expected segregating types		Results		pH measurements			
Genotype	Phenotype	No. of plants		No. of plants		Mean SD	
		F2	B1	F2	B1	F2	B1
Ph1-Hf1-Fl-	purple middle	187	28	164	26	5.49±0.09	5.46±0.07
ph1ph1Hf1-Fl-	purple bluish	90	20	77	16	5.96±0.09	5.90±0.10
Ph1-Hf1-flfl	purple reddish	75	24	65	20	5.51±0.08	5.47±0.09
ph1ph1Hf1-flfl	purple purplish	43	28	34	23	6.05±0.11	5.95±0.22
Ph1-hf1hf1Fl-	magenta middle	70	–	57		5.49±0.10	
ph1ph1hf1hf1Fl Fl	magenta bluish	0	–				
Ph1-hf1hf1flfl	magenta reddish	33	–	25		5.46±0.10	
ph1ph1fh1hflflfl	magenta purplish	0	–				
Total		498	100	422	85		
	F2			B1			
Segregation for	*Ph1* : 365 : 133	$P_{3:1}$ = 0.38		52 : 48		$P_{1:1}$ = 0.70	
	Hf1 : 395 : 103	$P_{3:1}$ = 0.04		–			
	Fl : 347 : 151	$P_{3:1}$ = <0.01		48 : 52		$P_{1:1}$ = 0.70	
Linkage *Ph1-Hf1*	: 262 : 103 : 133 : 0	$P_{2\times2}$ = <0.001		–			
Ph1-Fl	: 257 : 108 : 90 : 43	$P_{2\times2}$ = 0.56		28 : 24 : 20 : 28		$P_{2\times2}$ = 0.23	

Table 3. F2 and B1 of the cross V28 × M1; F1 $\frac{Ph2}{Ph2}\frac{Hf1}{hf1}\frac{Fl}{fl}$; B1: F1 × M51; genetical background *RtRtPh1Ph1Ph3Ph3Ph4Ph4*

Expected segregating types		Results		pH measurements			
Genotype	Phenotype	No. of plants		No. of plants		Mean SD	
		F2	B1	F2	B1	F2	B1
Ph2-Hf1-Fl-	purple middle	140	21	138	21	5.37±0.08	5.39±0.06
ph2ph2Hf1-Fl-	purple bluish	30	20	29	20	5.77±0.14	5.87±0.05
Ph2-Hf1-fl	purple reddish	45	22	45	22	5.43±0.07	5.41±0.04
ph2ph2Hf1-flfl	purple purplish	19	20	19	20	5.87±0.09	5.92±0.07
Ph2-hf1hf1Fl- *ph2ph2hf1hf1Fl* –	magenta middle[a] bluish	105	26	97	26	5.51±0.17	5.62±0.17
Ph2-hf1hf1flfl	magenta reddish	42	13	41	13	5.46±0.09	5.45±0.07
ph2ph2hf1hf1flfl	magenta purplish	11	15	11	15	5.82±0.17	5.85±0.11
Total		392	137	380	137		
		F2		B1			
Segregation for	*Ph2* : 227 : 60	$P_{3:1}$ = 0.12		56 : 55		$P_{1:1}$ = 0.93	
	hf1 : 234 : 158	$P_{3:1}$ = < 0.001		83 : 54		$P_{1:1}$ = 0.02	
	Fl : 275 : 117	$P_{3:1}$ = 0.03		67 : 70		$P_{1:1}$ = 0.79	
Linkage *Ph2-Hf1*	: 185 : 42 : 49 : 11	$P_{2\times2}$ = 0.93		43 : 13 : 40 : 15		$P_{2\times2}$ = 0.63	
Ph2-Fl	: 140 : 87 : 30 : 30	$P_{2\times2}$ = 0.04		21 : 35 : 20 : 35		$P_{2\times2}$ = 0.70	

[a] In this cross the colour classes magenta middle and magenta bluish could not be distinguished with certainty, therefore the number of plants belonging to these classes are summarized

In homogenates of *Ph1*-plants, a mean pH value of 5.48 was found, in *ph1ph1* plants, of 5.97. These values are independent of the genes *Hf1* and *Fl*, and so independent of the nature of the anthocyanins and amount of flavonol. Figure 1 A, B shows that there is hardly any overlap between the pH values of *Ph1*- and *ph1ph1* plants.

The gene Ph2

The results of the cross V28 × M1, given in Table 3, are about the same as in the previous cross. Because the gene *Ph2* is independent of the gene *Hf1* the colour types bluish and middle magenta are also found. These pale colour types are difficult to separate with certainty. Figure 1 C, D indicates that there is a small overlap between the pH values of *Ph2*- and *ph2ph2* plants. The middle (*Ph2*-) and bluish (*ph2ph2*) magenta types occupy an intermediate position between these groups. In this cross also there is no indication that the pH depends on the nature of anthocyanins or the amount of flavonol.

The gene Ph3

The results of the cross (V12 × M40) × R57 are presented in Table 4. In this cross the segregation for the gene *Ph3* deviates significantly from the expected 1 : 1 ratio,

Table 4. B1 of the cross V12 × M40; F1 $\frac{Ph3}{ph3}\frac{Hf1}{hf1}$; B1: F1 × R57; genetical background *RtRtPh1Ph1Ph2Ph2Ph4Ph4f f*

Expected segregating types		Results	pH measurements	
Genotype	Phenotype	No. of plants	No. of plants	Mean SD
Ph3ph3Hf1hf1	purple reddish	79	79	5.53±0.08
ph3ph3Hf1hf1	purple purplish	37	37	5.86±0.14
Ph3ph3hf1hf1	magenta reddish	71	71	5.52±0.10
ph3ph3hf1hf1	magenta purplish	26	26	5.88±0.11
Total		213	213	
Segregation for	*Ph3* : 160 : 63	$P_{1:1}$ = < 0.001		
	Hf1 : 116 : 97	$P_{1:1}$ = 0.19		
Lingage *Ph3-Hf1*	: 79 : 71 : 37 : 26	$P_{2\times2}$ = 0.43		

Table 5. F2 and B1 of the cross V12 × R62; F1 $\frac{Ph4}{ph4}\frac{Hf1}{hf1}\frac{Rt}{rt}$; B1: F1 × R62; gentical background *Ph1Ph1Ph2Ph2Ph3Ph3f1f1*

Expected segretating types		Results		pH measurements			
Genotype	Phenotype	No. of plants		No. of plants		Mean SD	
		F2	B1	F2	B1	F2	B1
Ph4-Hf1-Rt-	purple reddish	86	23	86	23	5.65±0.12	5.61±0.09
ph4ph4Hf1-Rt-	purple purplish	30	13	30	13	5.93±0.10	5.90±0.11
Ph4-Hf1-rtrt	grey 1 reddish	139	18	139	18	5.66±0.13	5.67±0.12
ph4ph4Hf1-rtrt	grey 1 purplish	38	26	38	26	5.89±0.10	5.83±0.14
Ph4-hf1hf1Rt-	magenta reddish	13	14	13	14	5.68±0.15	5.58±0.05
ph4ph4hf1hf1Rt-	magenta purplish	4	31	4	31	5.91±0.25	5.91±0.14
Ph4-hf1hf1rtrt	red 1 reddish	21	24	21	24	5.60±0.16	5.50±0.09
ph4ph4hf1hf1rtrt	red 1 purplish	11	41	11	41	5.91±0.11	5.82±0.12
Total		342	190	342	190		
	F2			B1			
Segregation for *Ph4*	: 295 : 83	$P_{3:1}$ = 0.76		79 : 111		$P_{1:1}$ = 0.03	
Hf1	: 293 : 49	$P_{3:1}$ = <0.001		80 : 110		$P_{1:1}$ = 0.04	
Rt	: 133 : 209	$P_{3:1}$ = <0.001		81 : 109		$P_{1:1}$ = 0.04	
Linkage *Ph4-Hf1*	: 225 : 34 : 68 : 15	$P_{2\times2}$ = 0.27		41 : 38 : 39 : 72		$P_{2\times2}$ = 0.03	
Ph4-Rt	: 99 : 160 : 34 : 49	$P_{2\times2}$ = 0.17		37 : 42 : 44 : 67		$P_{2\times2}$ = 0.03	

probably caused by a decreased viability of *ph3ph3* individuals. There is a small overlap between the pH values of *Ph3ph3* and *ph3ph3* plants (Fig. 1 E).

The difference in the nature of the anthocyanins (gene *Hf1*) has no influence on the pH value.

The gene Ph4

In Table 5 the results are given of the cross V12 × R62. In addition to the segregation of *Hf1*, which also segregates in the previous crosses, the gene *Rt* also segregates, resulting in new colour classes, grey 1 and red 1. Reddish and purplish types in general are difficult to separate in the colour class grey 1. The expected lower pH values for the red 1 types are not found in this cross; in preliminary experiments pH values of red 1 types were 0.2–0.3 pH units lower in purplish types as well as reddish types (Table 1). However, the pH values found in this cross are for purplish and for reddish types very homogenous.

The considerable overlap between the pH values of *Ph4-* and *ph4ph4* genotypes seen in Fig. 1 G, I, can be caused by the fact that the mean pH values of the reddish and purplish colour classes are close to each

Table 6. F2 of the cross R62 × W5; F1 $\frac{ph4}{Ph4}\frac{An1rt}{an1Rt}$; genetical background *Ph1Ph1Ph2Ph2Ph3Ph3f1f1*

Expected segregating types		Results	pH measurements	
Genotype	Phenotype	No. of plants	No. of plants	Mean SD
Ph4-An1-Rt-	magenta reddish	229	194	5.50±0.11
ph4ph4An1-Rt-	magenta purplish	143	133	5.68±0.08
Ph4-An1-rtrt	red 1 reddish	105		
ph4ph4An1-rtrt	red 1 purplish	68		
--an1an1--	white	143	135	5.74±0.11
Total		688		
Segregation for: *Ph4*[a]	: 334 : 211	$P_{3:1}$ = <0.001		
Rt[a]	: 372 : 173	$P_{3:1}$ = <0.001		
[b]		$P_{2:1}$ = 0.43		
An1	: 545 : 143	$P_{3:1}$ = 0.02		
Linkage *Ph4-Rt*	229 : 105 : 143 : 68	$P_{2\times2}$ = 0.84		

[a] Only in *An1-* plants

[b] Because cross-overs were never found between *Rt* and *An1* (so *an1an1* is *RtRt*), the segregation is also compared with a 2 : 1 ratio

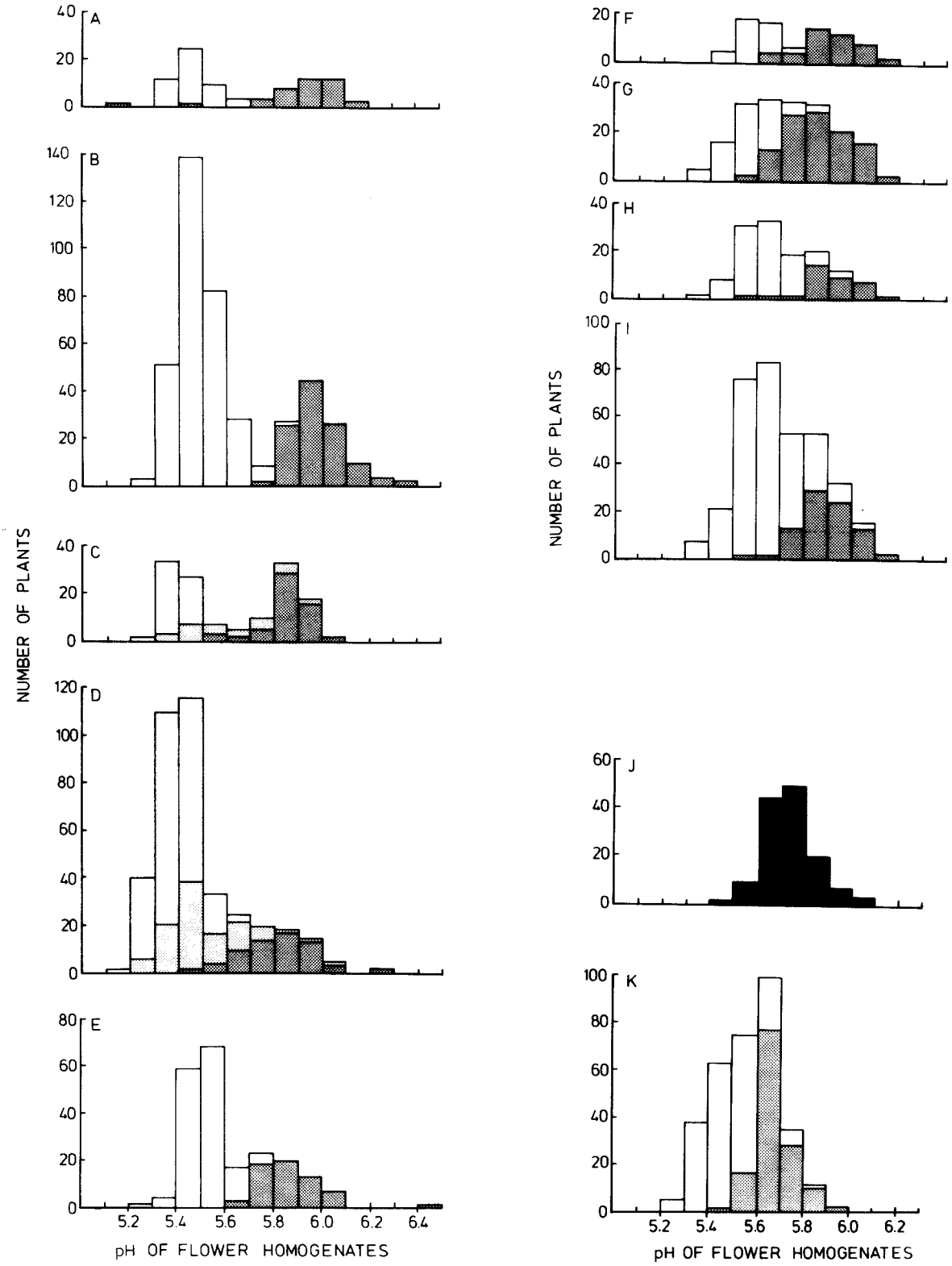

Fig. 1 A–K. pH of flower limb homogenates and segregation for *Ph* genes **A** B1 (V18 × M1) × V36 (*Ph1*); **B** F2 V18 × M1 (*Ph1*); **C** B1 (V28 × M1) × M51 (*Ph2*); **D** F2 V28 × M1 (*Ph2*); **E** B1 (V12 × M40) × R57 (*Ph3*); **F** B1 (V12 × R62) × R62 (*Ph4*), magenta + purple types; **G** all types; **H** F2 V12 × R62 (*Ph4*), magenta + purple types; **I** all types; **J** F2 R62 × W5 (*Ph4*); white types; **K** magenta types. □ all Ph genes dominant; ⊡ one pH gene homozygous recessive: ▒ magenta middle and magenta bluish types; ■ white types

other as compared with the crosses for the genes *Ph1, Ph2* and *Ph3*. However, if the classes grey 1 and red 1 are omitted (Fig. 1 F, H), the correlation between *Ph4* and the pH is more clear. It can also be concluded from this cross with *Ph4* that there is no (or very few) effect of the genes *Rt* and *Hf1* on the pH values.

In the cross R62×W5 there is a segregation in coloured and white flowering plants (Table 6). The mean pH values of the reddish and the purplish magenta types do not correspond completely with those found in the cross V12×R62. However it is clear that the mean pH value of the white flowering types (expected 3/4 *Ph4-* and 1/4 *ph4ph4*) is comparable with the pH values of magenta purplish types (Fig. 1 K, L). It can be concluded that the dominant allele of the gene *Ph4* has no effect in plants homozygous recessive for the gene *An1*.

The question remains whether the measured pH in flower limb homogenates is the same as the pH in the vacuole of a flower epidermis cell, and whether the observed differences in flower colour reflect changes in the pH of the vacuole.

To investigate this we have studied the effect of the pH on the spectrum of anthocyanins in flower limb homogenates. A complication is that anthocyanins in aqueous homogenates are unstable in the pH range 5.0–6.5, the homogenates discolour immediately by hydration of the anthocyanins. In a 4M NaCl solution anthocyanins are protected against hydration and are stable for more than 1 h (Goto et al. 1976). In Fig. 2 it is demonstrated that homogenates of magenta reddish and magenta purplish flowers do not differ in their behaviour in buffered 4M NaCl solutions. The absorption maximum shows the same shift toward higher wave lengths in solutions of a higher pH. Visually observed, the colour shifts in the solutions were the same as the difference in colour between reddish and purplish flowers. Thus, we feel that indeed the *Ph* genes are involved in maintaining the intravacuolar pH.

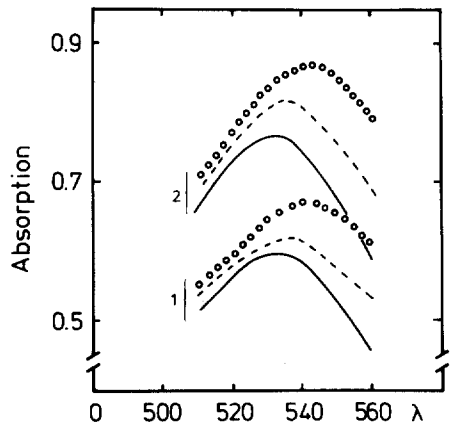

Fig. 2. Absorption spectra of homogenates of magenta purplish (1) and magenta reddish (2) flower limbs in buffered 4M NaCl solutions. —— pH 5.40 λ max 532 nm; - - - pH 5.82 λ max 535 nm; ○ ○ ○ pH 6,33 λ max 542 nm

Discussion

In the preceding, four complementary genes are described, each with two common effects: (1) in the presence of the dominant allele of these genes the colour of the flower limb is more reddish and (2) the pH of the flower limb homogenate is somewhat lower compared with that of the recessive allele. It has been shown, that both the reddening effect of the genes and the pH measured in homogenates are independent of the structure of the anthocyanins and of the concentration of flavonol in the flower limb.

In limb homogenates of white flowering *an1an1* plants a pH was measured comparable with the pH found in purplish types, independent of the genotype with respect to *Ph4*. This state was also found if *Ph1* was involved, implying that the *Ph* genes only have an effect in plants dominant for *An* genes. This could be explained either by a controlling effect of *An1* on the *Ph* genes (compare Tabak et al. 1981), or by the accumulation of anthocyanins being a prerequisite for the expression of *Ph* genes. In this respect it should be interesting to investigate segregation of *Ph* genes in white flowering mutants homozygous recessive for another anthocyanin gene (*An6*).

Very low pH values in anthocyanin containing vacuoles have been reported in the literature (pH 2.7 in *Vitis;* Moskowitz and Hrazdina 1981). Our values, however, fit well into the range of pH values reported by Stewart et al. 1975. In their paper a pH value of 5.5 is mentioned for the *Petunia* cultivar 'Maytime', which in our classification belongs to the flower class red 1 reddish.

No information is available on the molecular basis of the effect of *Ph* genes.

In general, the pH inside an organelle is the result of either a Donnan equilibrium across the outer membrane or an enzyme dependent process in which protons are actively transported across the membrane into the organelle. Furthermore, possible intravacuolar enzyme activities leading to the generation of protons may contribute to a low pH inside the vacuole.

We feel that mutants, as described in this paper, may be of interest in studies concerning the mechanism of maintaining the intravacuolar pH.

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