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Inheritance of the *An2* Gene and Epistatic Interactions in *Petunia exserta* x *P. axillaris* Hybrids

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Abstract. A regulatory gene, *An2*, controls structural genes within the flavonoid biosynthetic pathway. The inheritance of *An2* expression in crosses between *P. axillaris* (*an2*) and *P. exserta* (*An2*$^+$) was studied. Floral pigmentation was quantitatively inherited and involved the expression of a single regulatory gene (*An2*) and three structural genes (*Hf1, An6* and *Fl*). White flowers were produced in *an2*$^-$ genotypes; while pigmented flowers were produced in *An2*$^+$ genotypes. The intensity of pigmentation was determined by the interaction of *An2* with *An6, Hf1* and Fl, as well as substrate competition between the *An6* and *Fl* encoded enzymes.

The flavonoid biosynthetic pathway is well understood in the genus *Petunia* (Holton and Cornish, 1995; Wiering, 1974; Winkel-Shirley, 2001) and all of the enzymes and their corresponding genes have been studied in detail (Fig. 1). In *Petunia* flowers, the genes encoding the enzymes that are expressed early in the anthocyanin biosynthetic pathway (chalcone synthase, chalcone-flavone isomerase, flavanone 3-hydroxylase, etc.) are controlled by a different set of regulatory genes than those encoding the enzymes expressed late in the pathway (dihydroflavonol reductase, anthocyanin rhamnosyltransferase, anthocyanin methyltransferase, etc.) (Quattrocchio et al., 1993). At least four regulatory genes (*An1, An2, An4*, and *An11*) are required for the transcription of the genes expressed late in the pathway. *An1* encodes a basic helix-loop-helix (bHLH) transcription factor that is active in all parts of the flower (Spelt et al., 2000). *An2* and *An4* encode MYB-domain transcription factors (Quattrocchio et al., 1999). *An2* is active only within the petals, while *An4* is active only within the anthers. *An11* encodes a regulatory protein with five WD-repeat units that is active in all parts of the flower (de Vetten et al., 1997).

These regulatory genes operate in a complex regulatory hierarchy that is still not completely understood. The *An11* encoded cytoplasmic protein regulates the expression of *An2* and other nonanthocyanin related genes (de Vetten et al., 1997). It appears that *An11* links cellular and/or environmental signals with transcription of *An2*. However, *An2* does not directly regulate the transcription of any anthocyanin structural gene. *An2* controls the expression of *An1* which directly activates the transcription of the structural genes within the limb and tube (Spelt et al., 2000). Besides regulating anthocyanin biosynthesis, *An1*, *An2*, and *An11* also control vacuolar pH (Mol et al., 1998). *An1* (previously studied as *Ph6*) regulates the expression of the *Ph1* and *Ph2* structural genes that encode Na^+/H^+ exchanger proteins (*NHX1*) (Griesbach, 1998; Yamaguchi et al., 2001).

Petunia axillaris (Lamarack) Britton, Sterns et Poggenburg is the only *Petunia* species with white flowers. The lack of pigmentation is due to the absence of *An2* expression (Wijsman, 1983). In previous work (Mather and Edwardes,1943), the color of progeny between *P. axillaris* and *P. violacea* (=*P. integrifolia* (Hooker) Schinz et Thellung) varied depending upon the population of *P. axillaris* used. All of the F_1 plants produced flowers that were the same intensity of color as *P. integrifolia*. These plants, however, had purple (Munsell 1.3 RP 4.4/12.0) flowers versus the magenta (Munsell 7.6RP 4.9/13.6) of the *P. integrifolia* parent. Mather and Edwardes (1943) concluded that there must be at least a two gene difference between *P. axillaris* and *P. integrifolia*; however, segregation ratios did not fit any known inheritance pattern. The authors suggested the distortion in segregation resulted from the action of polygenes.

Another explanation for the distortion in the segregation ratios could be in the meiotic pairing between these species. If pairing is not normal, segregation ratios are distorted (Jackson, 1991). Several observations suggest this is occurring in *P. axillaris* x *P. integrifolia* hybrids. First, the hybrid can only be made with *P. axillaris* as the female parent (Mather, 1943). Second, meiotic abnormalities (univalents, laggards, unequal chromatid distribution, etc.) can be seen in the F_1 interspecific hybrid (Steere, 1932).

This paper describes the inheritance of the *An2* regulatory gene in crosses between *P. axillaris* and *P. exserta* Stehmann. *Petunia exserta* is a newly described species with red flowers that is closely related to *P. axillaris* (Stehmann, 1987). Both species are in the same taxonomic section of the genus; therefore, chromosome pairing is expected to be normal in the interspecific hybrids.

Materials and Methods

Petunia axillaris and *P. exserta* were obtained, respectively, from K.C. Sink at Michigan State University and J.R. Stehman at Universidada Federal de Minas Gerais, Brazil. Plants were grown and flowered at Beltsville, Md., in a greenhouse using standard cultural practices. The anthocyanins were extracted from fresh flowers by grinding in 1% (v/v) HCl in methanol. Extracts were reduced to dryness at 40 °C under reduced pressure. The residue was dissolved in 1% HCl-methanol and clarified by centrifugation at 10,000 g_n for 2 min.

The anthocyanins were characterized by HPLC (Waters Maxima 820 with 490E Visible/UV Detector) on a 7.8 × 300 mm column of 5 µ Bondapak C18 using a 30 min linear gradient of 0% to 10% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid, followed by a 10 min linear increase to 20% (v/v) acetonitrile and finally held at 20% (v/v) acetonitrile for an additional 10 min. Flow rate was 1.0 mL·min^{-1} and detection was by absorption at 540 nm. Anthocyanins were characterized by coelution with known standards and by acid hydrolysis (Griesbach et al., 1991).

The anthocyanin extracts were acid hydrolyzed at 100 °C in 3 N HCl for 1 h and hydrolyzed products characterized by HPLC on

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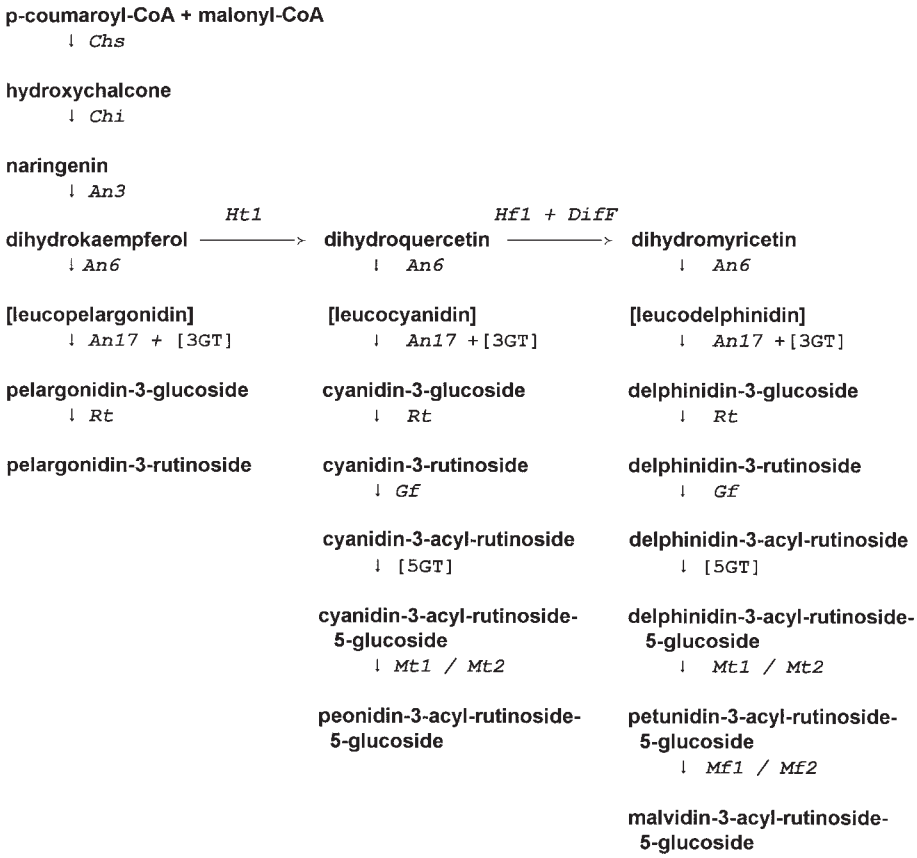


Fig. 1. Flavonoid biosynthetic pathway in *Petunia* (modified from Holton & Cornish, 1995 and Winkel-Shirley, 2001).

a 7.8 × 300 mm column of 5 µ Bondapak C18 using a 20 min linear gradient of 0% to 15% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid and held at 15% (v/v) for an additional 20 min. Flow rate was 1.0 mL·min^{-1} and detection was by absorption at 540 nm. Anthocyanidins were characterized by coelution with known standards (Griesbach et al., 1991).

Chromosomes from meiotic cells were examined using standard acetocarmine techniques (Berlyn & Miksche, 1976). Jackson's (1991) model for the genetic control of chromosome pairing was used to predict chiasmata distribution. The model assumes that if pairing is normal and is not influenced by outside genetic factors, then the distribution of chiasmata is nonrandom, and no univalents are expected. Broad sense heritability (H^2) was calculated using the method of Allard (1960): $H^2 = (V_{F2} - [V_{P1} + V_{P2} + V_{F1}]/3)/V_{F2}$.

Narrow sense heritability (h^2) was calculated using the method of Mather and Jinks (1982): $h^2 = (2V_{F2} - V_{B1} - V_{B2})/V_{F2}$.

Estimate of minimum number of genes was calculated using the method of Wright (1968): $n_{min} = (1.5 - 2h[1-h])\,(P_2 - P_1)^2/8(V_{F2} - V_E)$, where $h = (F_1 - P_1)/(P_2 - P_1)$, and $V_E = (V_{P1} + V_{P2} + 2V_{F1})/4$.

Scaling tests for the additive-dominance model were used to calculate $A = 2B_1 - P_1 - F_1$, $B = 2B_2 - P_2 - F_1$, $C = 4F_2 - 2F_1 - P_1 - P_2$, and $D = 2F_2 - B_1 - B_2$. If A = B = C = D = 0, then additive and dominance gene effects fully account for the observed variation (Mather and Jinks, 1982).

Results and Discussion

In this study, the F_1 hybrid between *P. exserta* and *P. axillaris* was completely fertile and exhibited a normal, nonrandom distribution of chiasmata (Table 1). Therefore, one could assume unrestricted gene flow between the two species and undistorted gene segregation. In addition, all of the genes (*An2*, *An6*, *Fl*, *Hf1*, *DifF, Mt*, and *Mf*) described in this study are unlinked (Cornu, 1984). The genotype of *P. exserta* is *An2 An6 fl Hf1 Mt2 mt1 mf1 mf2* and that of *P. axillaris* is *an2 An6 Fl hf1 Mt2 mt1 mf1 mf2* (Quattrocchio et al., 1999).

Petunia axillaris had white flowers with no detectable anthocyanins (data not shown). Flowers of *P. exserta* contained 13% delphinidin 3-glucoside, 57% delphinidin 3-rutinoside, 23.0% cyanidin 3-rutinoside and 7% petunidin 3-rutinoside as expected (Ando et al., 1999). The F_1 hybrid between *P. exserta* and *P. axillaris* contained 2% delphinidin 3-glucoside, 32% delphinidin 3-rutinoside, 7% cyanidin 3-rutinoside, 8% petunidin 3-caffeoylrutinoside-5-glucoside, 44% petunidin 3-coumaroylrutinoside-5-glucoside, 2%

Table 1. The number of different meiotic associations at diakinesis and metaphase I within the F_1 hybrid of *Petunia axillaris* × *P. exserta*. The model of Jackson (1991) was used to predict chiasmata distribution. For NR: chi-square = 0, $P > 0.995$. For R: chi-square = 130, $P < 0.005$.

Cells	Observed			Expected (NR)[y]			Expected (R)		
(no.)	I[z]	cII	oII	I	cII	oII	I	cII	oII
35	0	219	26	0	219	26	49	121	75

[z]I = univalent; cII = chain bivalent with 1 chiasmata; oII = circle bivalent with two chiasmata.
[y]NR = normal, nonrandom distribution; R = mutant, random distribution.

Table 2. The number of plants with either white or pigmented flowers within the F_2 and F_1-backcross populations of *Petunia axillaris* × *P. exserta*.

Generation	Plants with white flowers (no.)	Plants with pigmented flowers (no.)	Expected ratio	χ^2 value (probability)
F_2	46	113	1:3	0.0260 ($P \leq 0.9$)
F_1 × *P. exserta*	1	94	0:1	0.0105 ($P \leq 0.9$)
F_1 × *P. axillaris*	48	55	1:1	0.0069 ($P \leq 0.975$)

malvidin 3-caffeoylrutinoside-5-glucoside, and 5% malvidin 3-coumaroylrutinoside-5-glucoside.

The presence of pigmented flowers was simply inherited as a single dominant gene (*An2*) (Table 2); while, the inheritance of anthocyanin concentration was more complex (Table 3 and 4). Only those plants that had pigmented flowers (*An2* genotype) were used in subsequent analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants. The broad and narrow sense heritabilities for total anthocyanin concentration of plants with pigmented flowers (*An2* genotypes) were 0.97 and 0.24, respectively (Tables 5). Thus, gains from selection are possible, but they would be small and progress would be slow. Scaling tests (A = 0.64, B = 4.38, C = 10.34, and D = 2.96) suggested that part of the genetic variation in the intensity of pigmentation was due to epistasis or gene interaction. In *An2* genotypes, a minimum of 2.4 genes was calculated to be involved in anthocyanin concentration.

Mather and Edwardes (1943) concluded that there was at least a two gene difference between *P. axillaris* and *P. integrifolia*. They suggested that the genotypes of *P. axillaris* and *P. integrifolia* were *wwMM* and *WWmm* respectively, where *W*– determined pigmented-flowers (*W*– were pigmented and *ww* were white) and *M*– determined the type of color (*MM* were purple, M– were purple-magenta, and *mm* were magenta). In the F_2 population, plants were divided into nine classes: white, flushed white, very pale purple, pale purple, purple, very pale magenta, pale magenta, magenta or purple-magenta. To fit the two gene model, Mather and Edwardes concluded that nearly all of the pale, very pale and flushed white plants were genetically white (*ww*), but pigmented.

Wiering and de Vlaming (1984) concluded that a three gene model (*An2, Fl,* and *Hf1*) better fit the segregation ratios than a two gene model. In the three gene model, white flowers were only produced in *an2 Fl hf1* genotypes. All other *an2* genotypes produced weakly pigmented flowers. Strongly pigmented flowers were produced in *An2* genotypes. Even the three gene model for flower color expression is too simple, given the F_2 and backcross populations deviated from expected segregation patterns for three genes. Neither research group addressed the wide range in pigment intensity found in advanced generations. This data suggests that a four gene model can explain the segregation data and the differences in the intensity of pigmentation.

In this study, the concentration and type of anthocyanin varied considerable within plants that had pigmented flowers (*An2*

Table 3. Anthocyanin concentration of flowers from 91 plants in the F_2 population of *Petunia axillaris* x *P. exserta*. Anthocyanins are reported as the percentage of the total anthocyanin present. De-3-glu, delphinidin 3-glucoside; De-3-rut, delphinidin 3-rutinoside; Cy-3-rut, cyanidin 3-rutinoside; Pt-3-caf, petunidin 3-caffeoylrutinoside-5-glucoside; Mv-3-caf, malvidin 3-caffeoylrutinoside-5-glucoside; Pt-3-cou, petunidin 3-coumaroylrutinoside-5-glucoside; and Mv-3-cou, malvidin 3-coumaroylrutinoside-5-glucoside.

Plant	De-3-glu	De-3-rut	Cy-3-rut	Pt-3-caf	Mv-3-caf	Pt-5-cou	Mv-5-cou
1	0	85	8	7	0	0	0
2	0	16	0	16	41	13	14
3	0	44	6	27	23	0	0
4	0	25	11	14	25	7	18
5	0	33	0	23	32	0	12
6	0	17	14	14	29	14	12
7	0	27	0	16	27	12	18
8	0	46	0	28	13	0	13
9	0	51	7	17	6	0	19
10	0	68	0	18	0	0	14
11	8	52	14	18	8	0	0
12	0	62	0	7	0	31	0
13	0	48	0	14	10	8	20
14	0	17	26	8	0	49	0
15	0	28	13	0	0	59	0
16	0	53	18	7	0	22	0
17	0	23	20	7	7	7	36
18	0	8	0	15	41	15	21
19	0	9	0	6	42	24	19
20	0	14	6	11	44	11	14
21	0	72	11	10	0	7	0
22	0	24	0	30	21	12	13
23	13	79	0	8	0	0	0
24	0	6	13	9	46	13	13
25	0	20	8	17	26	11	18
26	0	26	22	24	21	0	7
27	0	22	35	17	19	0	7
28	0	34	6	18	21	10	11
29	0	43	10	18	17	0	12
30	0	0	13	7	0	80	0
31	0	14	8	15	39	10	14
32	0	68	32	0	0	0	0
33	0	23	7	7	0	63	0
34	0	0	7	0	0	93	0

Table 3 (**continued**). Anthocyanin concentration of flowers from 91 plants in the F_2 population of *P. axillaris* x *P. exserta*. Anthocyanins are reported as the percentage of the total anthocyanin present. De-3-glu, delphinidin 3-glucoside; De-3-rut, delphinidin 3-rutinoside; Cy-3-rut, cyanidin 3-rutinoside; Pt-3-caf, petunidin 3-caffeoylrutinoside-5-glucoside; Mv-3-caf, malvidin 3-caffeoylrutinoside-5-glucoside; Pt-3-cou, petunidin 3-coumaroylrutinoside-5-glucoside; and Mv-3-cou, malvidin 3-coumaroylrutinoside-5-glucoside.

Plant	De-3-glu	De-3-rut	Cy-3-rut	Pt-3-caf	Mv-3-caf	Pt-5-cou	Mv-5-cou
35	0	0	7	0	0	93	0
36	0	8	0	10	0	82	0
37	0	18	6	6	0	70	0
38	0	34	38	0	0	16	12
39	7	14	29	17	33	0	0
40	9	54	22	15	0	0	0
41	0	0	16	7	0	77	0
42	0	42	18	0	40	0	0
43	0	26	28	46	0	0	0
44	0	67	0	0	8	25	0
45	0	68	0	12	0	20	0
46	0	33	16	29	22	0	0
47	0	39	0	26	25	0	10
48	0	73	19	8	0	0	0
49	0	0	11	8	6	75	0
50	0	0	9	0	0	91	0
51	0	43	27	17	13	0	0
52	0	21	41	18	11	9	0
53	0	34	14	14	20	8	10
54	0	0	30	14	56	0	0
55	0	0	8	17	75	0	0
56	0	51	7	26	10	0	6
57	0	12	7	16	24	0	41
58	0	43	0	33	24	0	0
59	0	0	0	0	86	0	14
60	0	16	0	17	50	7	10
61	0	0	0	9	59	9	23
62	0	65	21	7	0	7	0
63	0	76	24	0	0	0	0
64	9	64	17	0	0	10	0
65	8	74	18	0	0	0	0
66	10	69	8	0	13	0	0
67	0	28	30	22	20	0	0
68	0	34	29	19	6	6	6
69	6	36	28	20	10	0	0
70	7	37	24	21	11	0	0
71	0	84	16	0	0	0	0
72	0	28	14	0	17	19	22
73	0	84	0	16	0	0	0
74	0	45	9	24	15	0	7
75	0	32	10	19	14	19	6
76	0	46	0	0	13	28	13
77	0	66	9	0	8	8	9
78	0	44	9	27	20	0	0
79	0	51	23	18	8	0	0
80	0	46	11	17	6	20	0
81	0	37	6	19	14	16	8
82	0	23	0	12	10	37	18
83	0	33	8	8	0	40	11
84	0	10	7	10	59	7	7
85	0	55	21	14	10	0	0
86	0	45	9	14	23	0	9
87	0	45	0	33	22	0	0
88	0	15	8	32	35	10	0
89	0	56	12	20	12	0	0
90	0	54	7	27	12	0	0
91	0	21	38	11	30	0	0

Table 4. Anthocyanin concentration of flowers from 60 plants in the *P. exserta* backcross population of *P. axillaris* × *P. exserta*. Anthocyanins are reported as the percentage of the total anthocyanin present. De-3-glu, delphinidin 3-glucoside; De-3-rut, delphinidin 3-rutinoside; Cy-3-rut, cyanidin 3-rutinoside; Pt-3-caf, petunidin 3-caffeoylrutinoside-5-glucoside; Mv-3-caf, malvidin 3-caffeoylrutinoside-5-glucoside; Pt-3-cou, petunidin 3-coumaroylrutinoside-5-glucoside; and Mv-3-cou, malvidin 3-coumaroylrutinoside-5-glucoside.

Plant	De-3-glu	De-3-rut	Cy-3-rut	Pt-3-caf	Mv-3-caf	Pt-3-cou	Mv-3-cou
1	4	21	52	10	2	2	9
2	3	72	16	9	0	0	0
3	0	50	44	6	0	0	0
4	4	62	27	5	0	2	0
5	3	57	35	3	0	2	0
6	0	6	60	9	13	0	12
7	0	64	34	2	0	0	0
8	0	20	66	14	0	0	0
9	0	27	58	15	0	0	0
10	3	33	55	0	9	0	0
11	7	57	22	5	0	0	9
12	0	18	49	8	0	4	21
13	4	27	40	15	0	0	14
14	0	27	28	16	14	4	11
15	7	31	22	15	15	0	10
16	9	44	20	15	3	3	6
17	16	67	9	8	0	0	0
18	12	63	14	8	0	3	0
19	6	41	36	4	0	0	13
20	8	45	31	6	0	0	10
21	0	25	49	11	0	0	15
22	3	29	35	16	0	4	13
23	7	48	12	26	0	2	5
24	14	68	10	8	0	0	0
25	11	63	19	7	0	0	0
26	11	50	13	26	0	0	0
27	10	35	7	23	25	0	0
28	8	38	10	22	18	0	4
29	10	43	16	22	0	0	9
30	6	60	28	6	0	0	0
31	11	57	18	14	0	0	0
32	13	66	10	11	0	0	0
33	9	40	22	17	0	0	12
34	7	31	11	21	26	0	4
35	6	31	22	9	8	3	21
36	13	50	33	4	0	0	0
37	9	44	12	22	13	0	0
38	14	37	8	24	17	0	0
39	11	60	18	11	0	0	0
40	3	22	40	8	15	0	12
41	9	45	30	5	0	0	11
42	10	38	35	4	0	0	13
43	3	16	47	7	13	0	14
44	12	68	8	12	0	0	0
45	11	32	8	24	25	0	0
46	15	62	15	8	0	0	0
47	5	39	29	15	0	0	12
48	7	22	22	15	19	4	11
49	6	27	24	17	18	0	8
50	9	53	14	24	0	0	0
51	4	11	56	4	0	0	25
52	8	31	18	18	11	4	10
53	11	77	4	0	0	0	8
54	18	13	51	18	0	0	0
55	0	10	46	19	0	6	19
56	4	32	26	16	10	0	12
57	7	53	32	8	0	0	0
58	11	39	14	32	0	0	4
59	9	40	15	24	0	0	12
60	12	46	20	10	0	0	12

Table 5. Descriptive statistics of the anthocyanin content of flowers from plants within populations of the parents, F_1, F_2, and backcross hybrids of *Petunia axillaris* x *P. exserta*. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis can not be measured in a*n2* plants.

Plant	Mean[z]	Variance
P. exserta	9.30	0.34
F_1	7.86	0.26
F_2	3.52	5.81
F_1 x *P. axillaris*	1.74	2.91
F_1 x *P. exserta*	8.26	10.11

[z]ng·mg^{-1} dry weight.

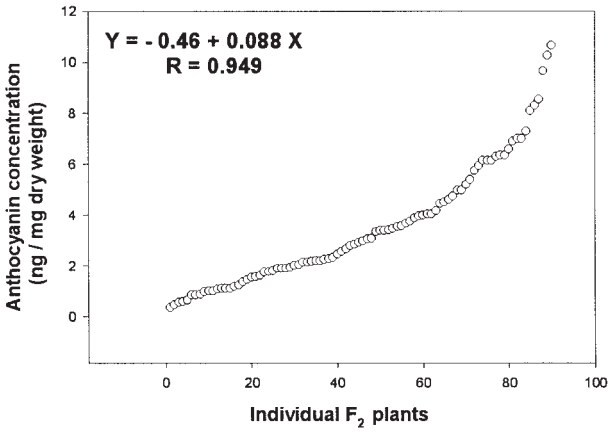
genotype) (Tables 3 and 4; Fig. 2, 3 and 4). In the F_2 population, plants with flowers containing the highest concentrations of petunidin had the lowest concentrations of malvidin and delphinidin (Fig. 5). In addition, F_2 plants with flowers containing the highest concentrations of total anthocyanin had the highest relative concentrations of petunidin (Fig. 6). Flowers from the four plants with the highest total anthocyanin concentrations contained over 70% petunidin.

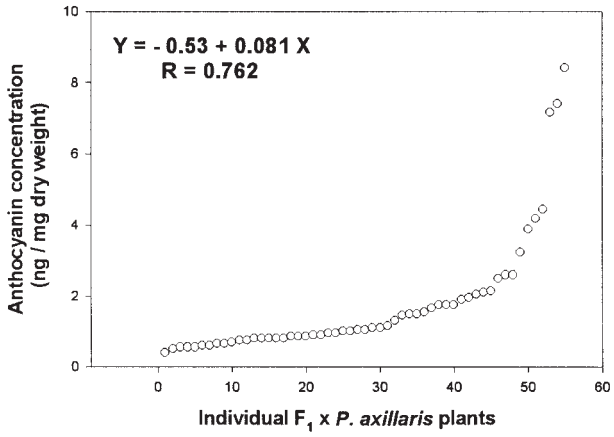
These data suggest that both *P. exserta* and *P. axillaris* had a *Mt2 mt1 mf1 mf2* genotype. There are four different anthocyanin-O-methyltransferase genes in *Petunia* (*Mt1, Mt2, Mf1,* and *Mf2*) (Jonsson et al., 1983; 1984). Each gene controls a distinct and independent enzyme that is capable of methylating both the 3' and 5' positions on the anthocyanin molecule (Fig.1). Each enzyme, however, has a distinct substrate specificity. The *Mf1* (8 μM) and *Mt2* (6 μM) encoded enzymes have ≈3-fold lower K_m values for cyanidin and petunidin glycosides as substrates than the *Mf2* (21 M) and *Mt1* (25 μM) encoded enzymes. Each enzyme also has a different efficiency in methylating delphinidin glycosides—*Mf1* (175 pkat·mg^{-1} protein), *Mf2* (100 pkat·mg^{-1} protein), *Mt1* (60 pkat·mg^{-1} protein), and *Mt2* (30 pkat·mg^{-1} protein). When delphinidin glycosides are the substrates, the *Mt* encoded enzymes produce mainly petunidin derivatives; while the *Mf* encoded enzymes produce mainly malvidin derivatives. There is, however, a differential effect on substrate inhibition. High concentrations of delphinidin glycosides reduce the amount of malvidin derivatives produced, but not the amount of petunidin derivatives produced. In addition, a dosage effect was suggested for *Mf/Mt* gene expression. As the number of *Mf/Mt* genes increase, the relative concentration of malvidin derivatives also increases.

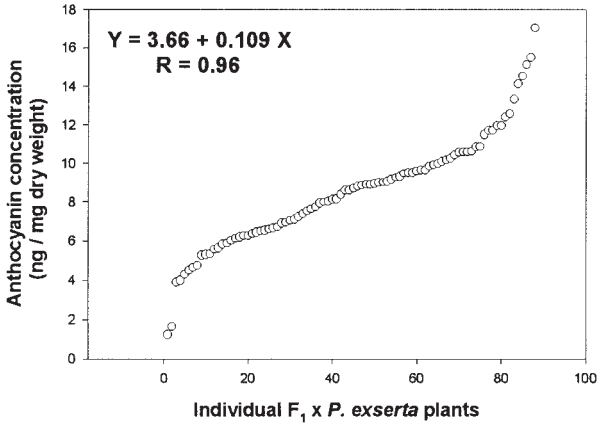
Of the F_2 plants with pigmented flowers, about 10% had flowers containing over 60% malvidin (Fig. 3). The plants that had high relative concentrations of malvidin had low concentrations of total anthocyanin (Fig. 6). These facts are consistent with the hypothesis that *P. exserta* and *P. axillaris* had a *mf1 mf2* genotype. In *mf1 mf2* genotypes, high concentrations of petunidin glycosides coupled with low concentrations of delphinidin glycosides allow the synthesis of malvidin derivatives (Farcy and Cornu, 1979; Jonsson et al., 1984). Those plants that had flowers with relative high malvidin concentrations had low relative delphinidin and high relative petunidin concentrations (Table 3).

Total anthocyanin concentration (*An2* expression) was negatively correlated to malvidin production (*Mf* expression) (Fig. 6). Plants expressing the highest total anthocyanin concentrations had the lowest malvidin concentration. Thus, it appears that *An2* negatively controlled the expression of *Mf*. The *An2* gene is known to control the expression of the *Mt* and *Mf* genes (Gerat et al., 1984). A controlling element was used to silence the *An2* gene in a *mf1 mf2* genotype (Farcy and Cornu, 1979). The *an2* sectors were not white, but contained ≈15-fold less anthocyanin than the *An2* sectors. In addition, the relative concentration of the various anthocyanins also differed in the sectors. In *An2 mf1 mf2* sectors,

Fig. 2. The total anthocyanin concentration in flowers from plants within the F_2 and F_1 backcross populations of *Petunia axillaris* x *P. exserta*. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants.







there was 5% delphinidin, 75% petunidin and 20% malvidin; while in *an2 mf1 mf2* sectors there was 65% delphinidin, 25% petunidin and 10% malvidin.

An2 also controls the expression *An6, An13, Fl,* and *Hf1* (Fig. 1) (Quattrocchio et al., 1999). *Hf1* encodes flavonoid 3',5'-hydroxylase, which is a cytochrome P450-dependent monooxygenase (de Vetten et al., 1999). This enzyme requires the presence of an additional protein (cytochrome b_5) encoded by *DifF*. Cytochrome b_5 acts as the electron donor between NADPH and cytochrome P450-dependent monooxygenase.

The conversion of dihydroflavonols to anthocyanins requires the concerted action of three enzymes (Saito et al., 1999). The first enzyme (dihydroflavonol reductase) is encoded by *An6* and catalyzes the conversion of dihydroflavonols to leucoanthocyanins (Huitts et al., 1994). The second enzyme (anthocyanidin synthase, a 2-oxoglutarate-dependant oxygenase) is encoded by *An17* and converts leucoanthocyanins into 3-flaven-2,3-diols (Weiss et al., 1993). The last enzyme (anthocyanin glucosyltransferase) creates the anthocyanin-3-glucoside (Kho et al., 1978). The anthocyanin glucosyltransferase gene has not yet been identified in *Petunia*.

Fl encodes flavonol synthase which is a 2-oxoglutarate-dependant oxygenase (Holton et al., 1993). In *Fl* genotypes, quercetin glycosides accumulate at the expense of cyanidin-based anthocyanins (Wiering and De Vlaming, 1984). To a lesser extent, myricetin glycosides accumulate at the expense of delphinidin-based anthocyanins in *Fl* genotypes.

The K_m of an enzyme does not always directly reflect activity in vivo. For example, if the dihydroflavonol reductase K_m for dihydroquercetin was lower than that for dihydromyricetin, then one would expect a higher concentration of delphinidin derivatives than cyanidin derivatives when both substrates are present at the same concentration. However, the opposite could occur if the flavonol synthase K_m for dihydromyricetin was higher than that of the dihydroflavonol reductase K_m for dihydromyricetin and if the flavonol synthase K_m for dihydroquercetin was lower than that of the dihydroflavonol reductase K_m for dihydroquercetin. In this instance, dihydromyricetin would be converted to myricetin at the expense of delphinidin and dihydroquercetin would be converted to cyanidin at the expense of quercetin. The ratio of cyanidin to delphinidin would be the opposite of what one would expected based only upon the K_m for dihydroflavonol reductase.

The differences in the concentration of total anthocyanin were probably not due to differences in *An2* transcription. The data

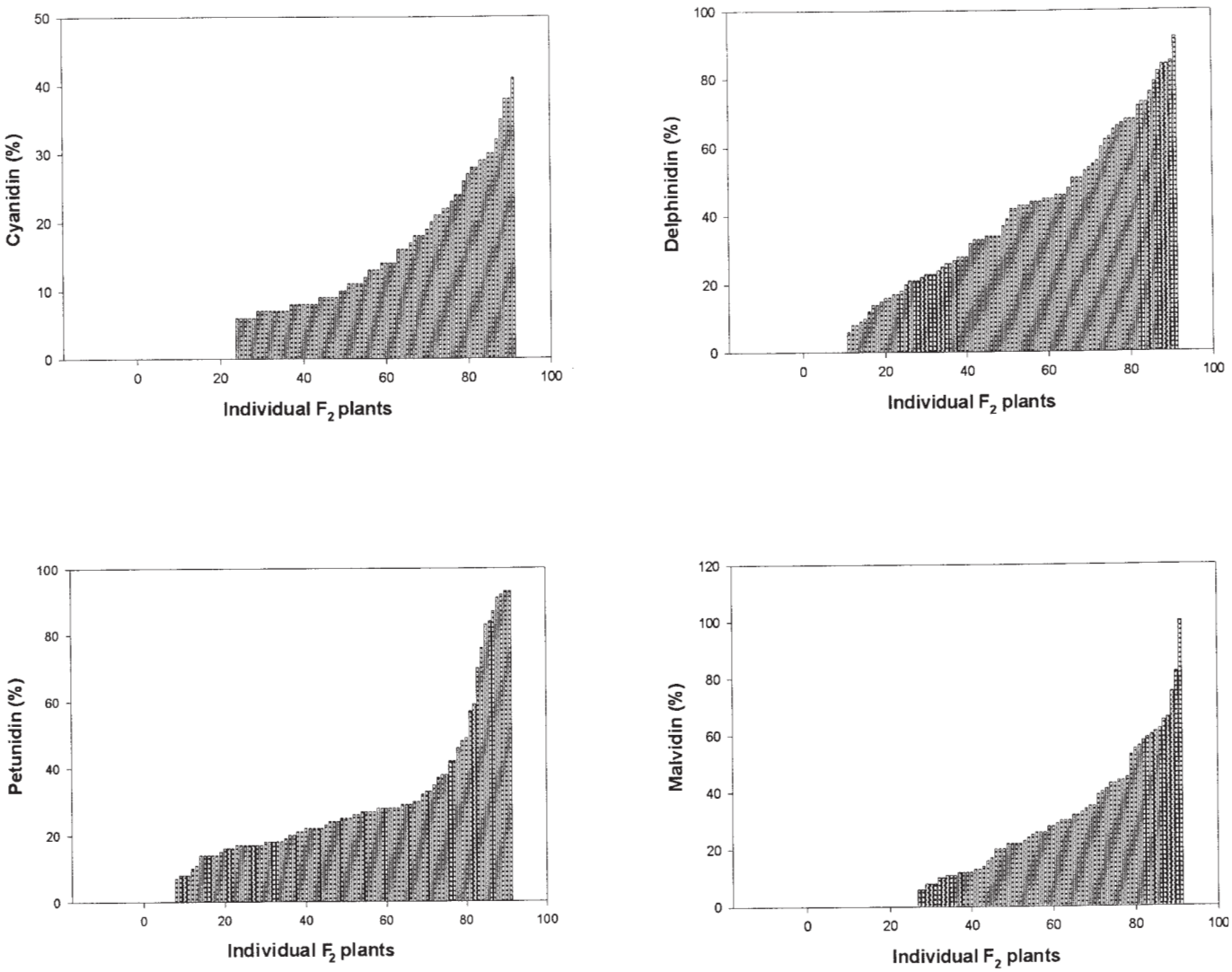


Fig. 3. The anthocyanin content of flowers from plants within the F_2 population of *Petunia axillaris* x *P. exserta*. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants.

suggest that the darkest and lightest flowers had the same level of *An2* expression. The difference in pigmentation intensity could have been the result of differential regulation of enzymes by *An2*. Two to three different genes are needed to explain the variation in the intensity of floral pigmentation. The simplest explanation involves the expression of *Fl, An6,* and *Hf1* (Fig. 7). In *fl* genotypes, full anthocyanin expression is expected leading to darkly pigmented flowers. In *Fl* genotypes, anthocyanin expression depends on the competition for substrate by the *Fl* and *An6* encoded enzymes. If the K_m value of the *An6* encoded enzyme is greater than that of *Fl* encoded enzyme, then more anthocyanin will be synthesized than flavonol. The ratio of the K_m values will determine anthocyanin concentration. *Hf1* influences anthocyanin concentration by changing the ratio of the two precursors. The *Fl* encoded enzyme has a higher K_m value for dihydroquercetin than dihydromyricetin (Wiering and De Vlaming, 1984). In *Fl Hf1* genotypes, the ratio of anthocyanin to flavonol is expected to be greater than in *Fl hf1* genotypes.

In conclusion, floral pigmentation was quantitatively inherited in these *Petunia* crosses and involved the expression of a single regulatory gene (*An2*) and three structural genes (*Hf1, An6,* and *Fl*). White flowers were produced in *an2* genotypes; while pigmented flowers were produced in *An2* genotypes. The intensity of pigmentation was determined by the interaction of *An2* with at least 2.4 other genes. The most likely candidate genes are *An6, Hf1* and *Fl.* The mechanism by which *An2* interacts with these genes is waiting investigation.

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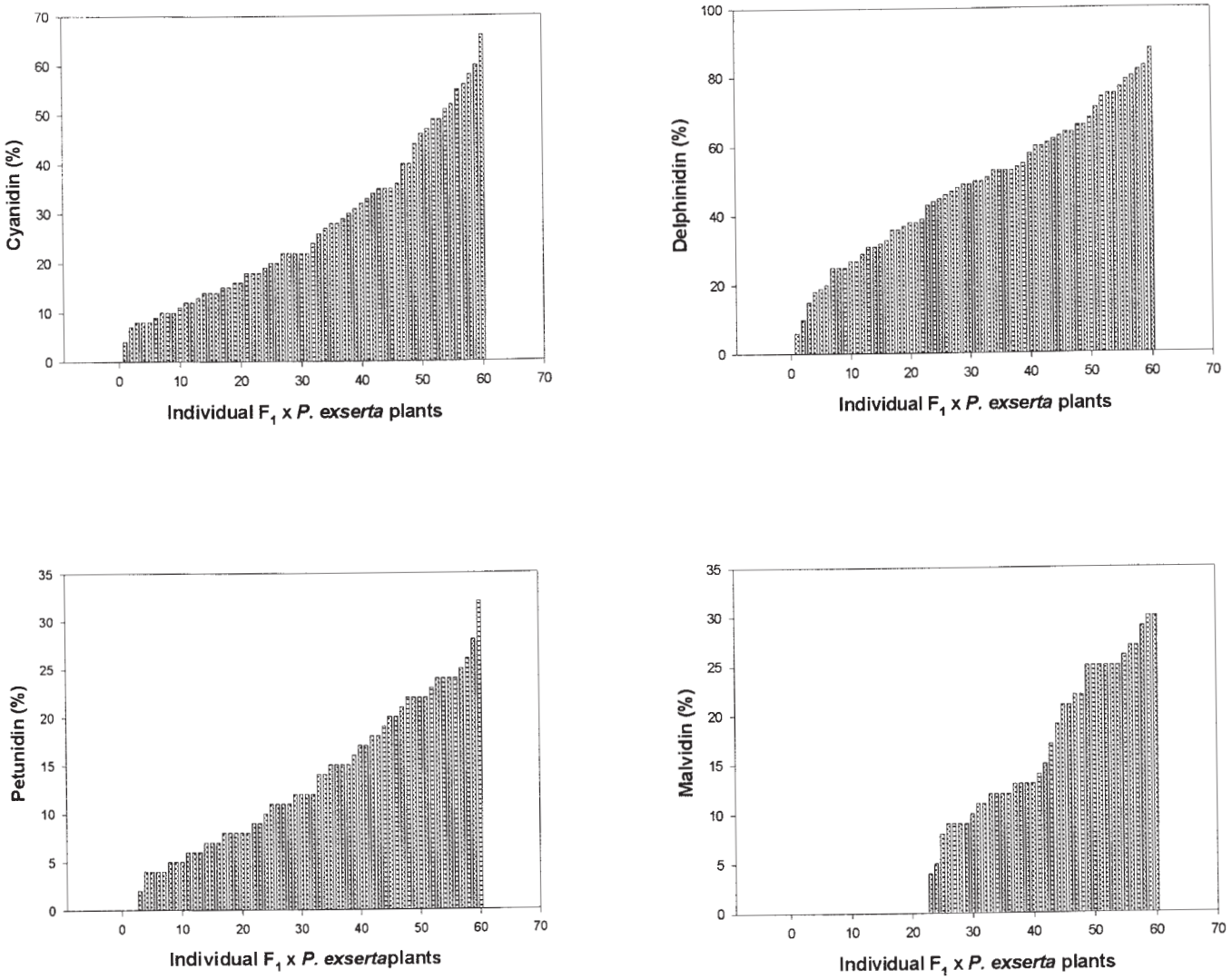

Fig. 4. The anthocyanin content of flowers from plants within the F_1 backcross to *Petunia exserta*. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants.

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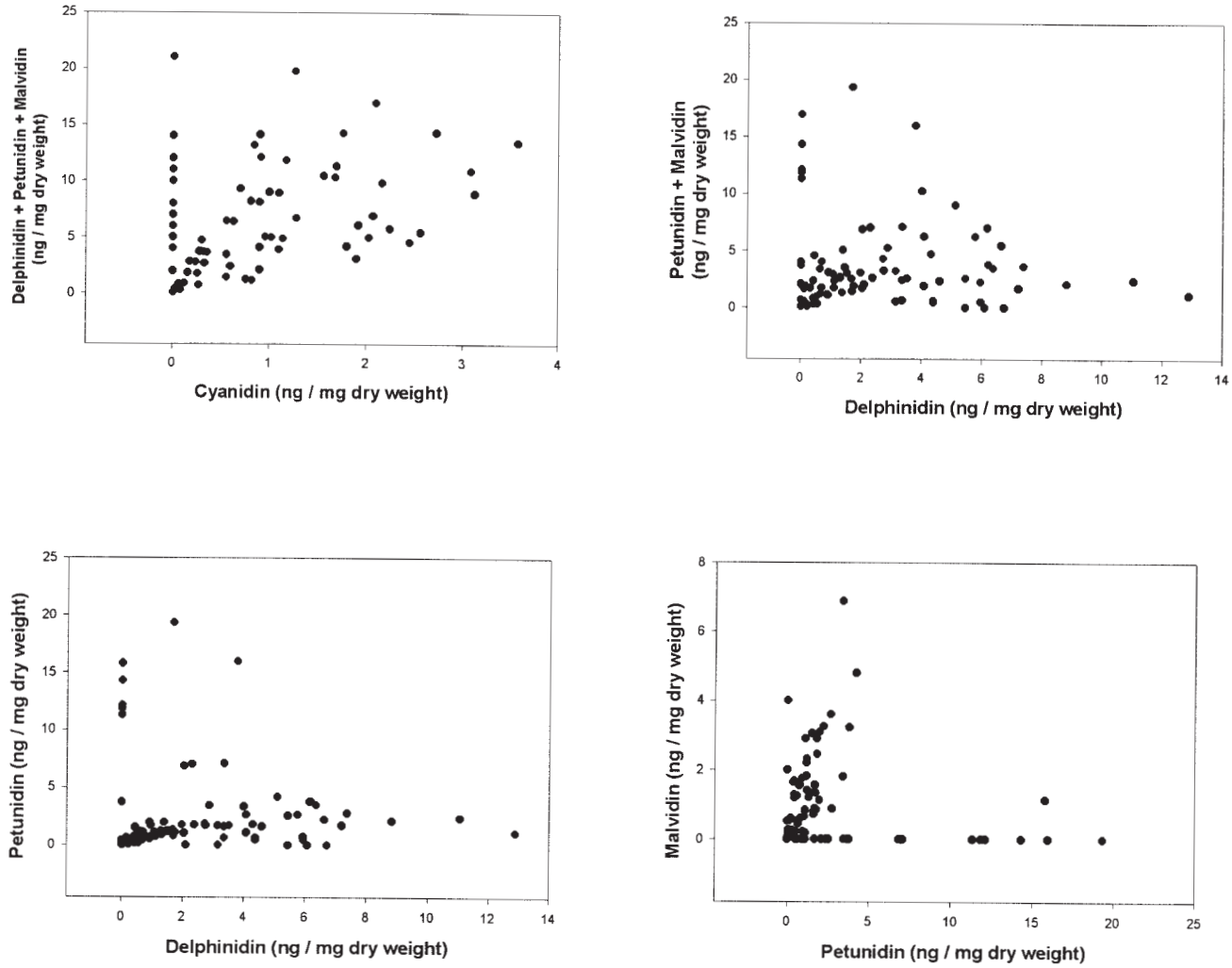

Fig. 5. Ratios of anthocyanin content of flowers from plants within the F_2 population of *Petunia axillaris* x *P. exserta*. Regression analysis was to compare specific anthocyanins within individual plants. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants.

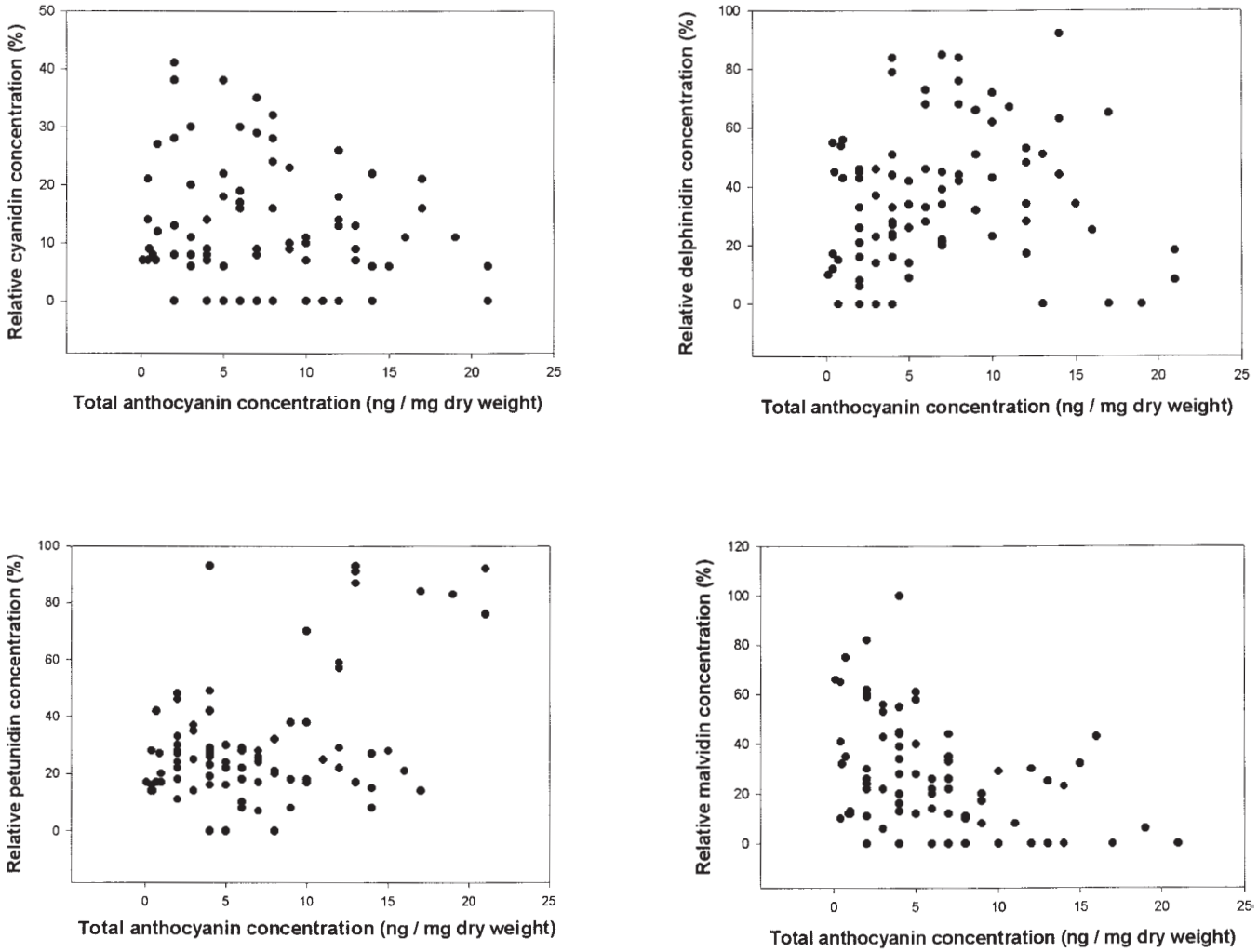

Fig. 6. Ratios of anthocyanin content of flowers from plants within the F_2 population of *Petunia axillaris* x *P. exserta*. Regression analysis was used to compare total anthocyanin concentration with the relative concentration of specific anthocyanins within individual plants. Only those plants that had pigmented flowers (*An2* genotype) were used in the analysis. Including plants with unpigmented flowers (a*n2* genotype) would bias the results because epistasis cannot be measured in a*n2* plants.

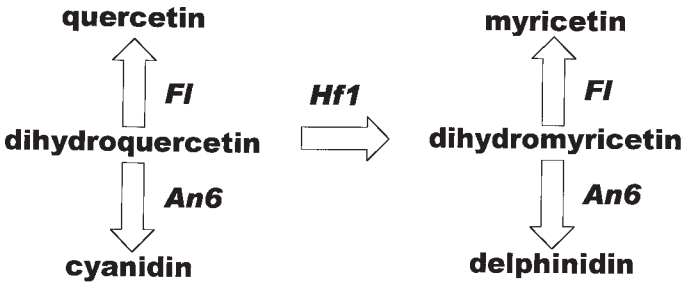

Fig. 7. A section of the flavonoid biosynthetic pathway leading to the synthesis of anthocyanins and flavonols from their common precursor.

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