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Isolation and Characterization of Anthocyanin Variants Originating from the Unstable System *an2-1* in *Petunia hybrida* (Hort.)

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Summary. Forty stable *an2-n* alleles, derived from the unstable system *an2-1*, have been tested for anthocyanin synthesis. All of them proved to be different from both the *An2* and *an2* natural alleles. Only two were distinct from the others which according to Duncan's multiple range test formed a group of overlapping populations. Amongst the variants isolated there was a large majority of light-coloured types. Regulation-like effects of the *an2-n* alleles on the subsequent genes of the anthocyanin pathway have been observed. A hypothesis concerning the nature of the genetic events occurring at the *An2* locus is discussed.

Key words: *Petunia hybrida* – Genetic instability – Anthocyanin synthesis – Allelic series

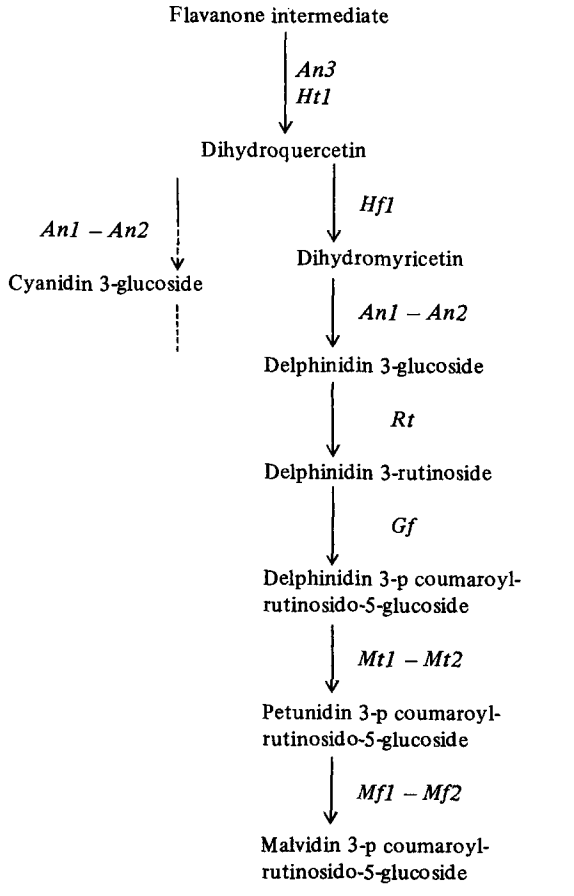
Introduction

The existence of allelic series controlling anthocyanin production in plants has been known for a long time. In general they seem to be linked to a quantitative rather than to a qualitative action on the anthocyanin synthesis, the R^r series for aleurone colour in maize grain (Stadler 1948, 1951) being an example. However they can also govern the distribution of these pigments in different organs, for example the *B* locus in potato (Dodds and Long 1956), or in different floral parts, for example the *Pal* locus in *Antirrhinum majus* (Fincham and Harrisson 1967).

Genetic instability provides a very important source of alleles at the concerned locus. Emerson had already reported it in 1929 for the mutable allele p^{vv} in maize. More recently, the characteristics of alleles derived from unstable genes have been studied by Reddy and Peterson (1976) for the *A2* locus in maize, and by Bianchi (1978) for the *An1* locus in petunia.

The unstable system *an2-1* in petunia (Cornu 1977) is characterised by the production of a large number of sectors with colours intermediate between the dominant and recessive phenotypes. With Wiering's (1974) and Kho et

Table 1. Genetic control of anthocyanins biosynthesis in *Petunia hybrida* (Hort.) according to Wiering (1974), Kho et al. (1977), Tabak et al. (1978)



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al.'s (1977) studies the role of the *An2* gene in the pathway of anthocyanin biosynthesis (Table 1) in petunia flowers now seems to be well established. Recently Kho et al. (1978) observed that the *An1* and *An2* genes influence the activity of UDP-glucose: cyanidine 3-0-glucosyltransferase.

The possibility of obtaining sexually stable variants corresponding to very diverse anthocyanin production prompted the present quantitative and qualitative study of anthocyanin synthesized under the control of isolated *An2* alleles in petunia. A precise analysis of how these new alleles function should lead to a better interpretation of both the role and the structure of the *An2* locus, and thus give some insight into the nature of the unstable system involved.

Material and Methods

The variant material used was derived from the *a1* unstable system (Cornu 1977). Following Wiering's (1974) terminology, it will now be referred to as *an2-1*.
The variants were obtained from the cross:

$$\frac{an2\text{-}1 \quad rt}{an2 \quad Rt} \times \frac{an2 \quad Rt}{an2 \quad Rt} \text{ (TL-h-1 line)}$$

(The *An2* and *Rt* loci are situated on the VI chromosome and are very closely linked (recombination level : 1‰). The parents of this cross were such that all the descendants were homozygotes for the genes *An1, An3, Hf1, Gf, Mt1, Mt2, mf1* and *mf2*. In this way the pathway of anthocyanin biosynthesis, as it is known at present, should lead to the production of petunidin (3p-coumaroyl-rutinosido-5-glucoside) as the main pigment. The harvests from this cross, consisting of more than 100,000 seeds, were mixed and 10% of these seeds were taken for sowing in order to avoid selecting the same variant too frequently in the offspring. Fifty variants, characterised by a stable colour different from the 2 parents, were isolated from these 10,000 daughter plants. Genetical analysis of the variants showed that they had the genotype constitution $\frac{an2\text{-}n \; rt}{an2 \quad Rt}$, *an2-n* representing the new alleles obtained from the unstable system. The characteristics of each of these alleles are stable and can be transmitted; the *an2-n* genes remain linked to the recessive gene *rt* in the same way as the original unstable allele *an2-1*. Pigments of 40 variants were analyzed using triplicate samples from each variant. The recessive homozygote *an2/an2* and the heterozygote *An2/an2* served as controls. All plants were heterozygous for the locus *Rt*.

Plants were grown under controlled conditions at 20°C with a 16 hour day. About 100 corolla limbs were collected from each plant when the flowers were fully developed. These were then dried at room temperature under vacuum in the presence of NaOH, ground into a powder and steeped in methanol/0.1% HCl overnight at −18°C. The extract was filtered through a glass microfilter, the residue washed with methanol/0,1% HCl and the total extract dried under vacuum at 35°C. The dried extract was hydrolysed in 2N HCl at 100°C for 30 min, then cooled at 0°C. The aglycones released by hydrolysis were extracted in a known volume of isoamylic alcohol and stored at −18°C. All operations were carried out under reduced light conditions.

The aglycones were analysed quantitatively by separating known quantities of extracts on thin layer cellulose chromatographic plates using a slightly modified Nybom (1964) solvent (HCOOH : 25% HCL:H_2O, 10:1:3, v/v). The chromatograms were dried in the dark at room temperature and the separated pigments were eluted in methanol/0.1% HCl immediately to avoid adsorption onto the cellulose. An Eluchrom Camag was used to elute the pigment spots quantitatively and the optical densities of the eluates were measured at 545nm (Beckmann Acta III spectophotometer). The results were expressed as quantity of delphinidin, petunidin and malvidin per 100 µg dried plant material (3 repetitions per plant and 3 plants per allele).

Anthocyanins present in extracts were analyzed qualitatively by separation on Macherey Nagel 300N cellulose plates using Metche's solvent (acetic acid : HCl : H_2O, 3:1:20, v/v). The marker pigments were supplied by P. de Vlaming (Amsterdam Genetics Institute). Three different classes of pigments could be clearly defined using this system : delphinidin 3 glucoside at Rf 0.13, delphinidin 3 rutinoside at Rf 0.30 and 3-5 substituted pigments (delphinidin, petunidin, malvidin, 3 p-coumaroyl-rutinosido-5-glucoside) at Rf 0.42 to 0.45. The anthocyanidin of pigments migrating at Rf 0.13 and 0.30 was confirmed as being delphinidin and all 3 types of anthocyanidin were found in the pigments migrating at Rf 0.42. The quantity of the 3 different classes of pigments in each extract was assessed visually and expressed as a percentage of the total pigments in the extract.

Results

1 Anthocyanin Synthesizing Ability of Isolated Variants

The isolated variants, which are each characterised by a new stable allele at the *An2* locus (general formula *an2-n/an2*), varied greatly in their capacity to synthesize anthocyanins (Table 2). They were all significantly different from the dominant allele *An2* and the recessive homozygote control *an2/an2*. No case of reversion towards the *An2* allele was observed. The synthesizing ability of these new alleles, under the present experimental conditions, were highly inferior to these of the *An2* control. This was

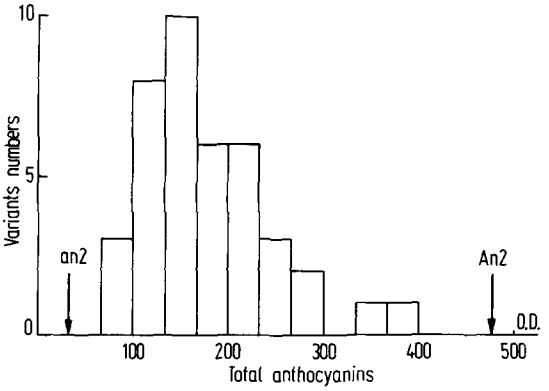

Fig. 1. Distribution of variants derived from the *an2-1* system in relation to their anthocyanin synthesizing ability

true even for the alleles *an2-189* and *190* which were significantly superior to all the others.

The majority of the isolated variants were light-coloured types: 80% of them had an anthocyanin synthesizing ability which was less than 50% of that of the original *An2* control (Fig. 1). There seemed to be a marked predominance of alleles having an activity of about 30% of that of *An2*.

Table 2. Qualitative and quantitative analysis of anthocyanins synthesized by plants with *an2-n* alleles

Alleles	Total anthocyanins* sum O.D. aglycones		O.D. aglycones**			% repartition of different anthocyanins***		
			Dp	Pt	Mv	DP-3G	DP-3RG	3-5 substituted pigments
An2	0.477	a	0.030	0.358	0.089	0	0 – 5	95 – 100
an2 – 190	0.386	b	0.023	0.281	0.082	0	0 – 5	95 – 100
an2 – 189	0.362	b	0.026	0.266	0.070	0	0	100
an2 – 157	0.282	c	0.026	0.209	0.047	0	0 – 5	95 – 100
an2 – 194	0.272	c	0.017	0.198	0.057			
an2 – 175	0.259	c d	0.040	0.185	0.034	0	0 – 5	95 – 100
an2 – 185	0.255	c d e	0.034	0.185	0.036	0	0 – 5	95 – 100
an2 – 167	0.243	c d e f	0.042	0.171	0.030			
an2 – 170	0.224	d e f g	0.038	0.159	0.027	0 – 5	10	85 – 90
an2 – 191	0.221	d e f g h	0.041	0.154	0.026			
an2 – 174	0.218	d e f g h	0.035	0.155	0.028			
an2 – 184	0.213	d e f g h i	0.051	0.139	0.023			
an2 – 187	0.209	e f g h i j	0.043	0.142	0.024	0 – 5	10	85 – 90
an2 – 154	0.207	f g h i j	0.035	0.146	0.026			
an2 – 192	0.191	g h i j k	0.042	0.125	0.024			
an2 – 181	0.186	g h i j k l	0.036	0.127	0.023			
an2 – 182	0.176	h i j k l m	0.041	0.115	0.020	0 – 5	10	85 – 90
an2 – 156	0.175	h i j k l m	0.044	0.112	0.019			
an2 – 179	0.175	h i j k l m	0.037	0.117	0.021			
an2 – 166	0.169	i j k l m n	0.034	0.115	0.020	0	0 – 5	95 – 100
an2 – 158	0.165	j k l m n	0.075	0.078	0.012	10	30	60
an2 – 171	0.152	k l m n o	0.040	0.097	0.015			
an2 – 193	0.150	k l m n o	0.054	0.082	0.014	10	20	70
an2 – 195	0.150	k l m n o	0.067	0.070	0.013	10	30	60
an2 – 161	0.145	k l m n o	0.050	0.083	0.012			
an2 – 178	0.143	k l m n o p	0.050	0.080	0.013			
an2 – 180	0.140	l m n o p q	0.079	0.051	0.010	20	40	40
an2 – 165	0.136	m n o p q	0.050	0.073	0.013	10	30	60
an2 – 152	0.135	m n o p q	0.057	0.068	0.010	10	30	60
an2 – 159	0.133	m n o p q	0.057	0.064	0.012			
an2 – 177	0.133	m n o p q	0.069	0.054	0.010	10	40	50
an2 – 163	0.131	m n o p q	0.053	0.067	0.011	10	30	60
an2 – 173	0.131	m n o p q	0.054	0.065	0.012	10	30	60
an2 – 155	0.123	n o p q r	0.050	0.063	0.010			
an2 – 168	0.122	n o p q r	0.049	0.062	0.011	10	30	60
an2 – 153	0.112	o p q r	0.054	0.049	0.009			
an2 – 172	0.112	o p q r	0.050	0.053	0.009	10	30	60
an2 – 162	0.106	o p q r	0.044	0.052	0.010	10	30	60
an2 – 164	0.096	p q r	0.040	0.046	0.010	10	30	60
an2 – 169	0.093	q r	0.046	0.040	0.007	10	40	50
an2 – 160	0.084	r	0.037	0.039	0.008	10	40	50
an2 –	0.034	s	0.022	0.009	0.003	50	30	20

* Values followed by the same letter are not significantly different from each other (P = 0.05) according to Duncan's multiple range test

** Dp = delphinidin, Pt = petunidin, Mv = malvidin, mean values for extracts of 3 plants $\frac{an2\text{-}nrt}{an2RT}$ obtained from the crossing of each variant *an2-n* by *TLh1*

*** Dp-3G = delphinidin 3-glucoside, Dp-3RG = Delphinidin 3-rutinoside, 3-5 substituted pigments = Delphinidin, Petunidin, Malvidin (3p-coumaroyl-rutinosido-5-glucoside)

0 = not detectable by the method used

2 *Production of Different Types of Anthocyanidins*

The genotype of the plants should normally lead to the accumulation of petunidin as the main pigment (Table 1). However, quantitative analysis of the 3 aglycones shows that this is not always the case. In the light-couloured types, where the total pigment content was less than 35% of that of the *An2* control, there was a high relative quantity of delphinidin (Table 2). The amount of delphinidin decreased slightly when the total anthocyanin content increased (coeff. of correlation, r = −0.36 significant). Petunidin was the main pigment in plants where the total pigment content was greater than 35% of that of the *An2* control. There was a strong and positive correlation between petunidin and total pigment content (r = 0.99).

Even though the genes for methylation *Mf*, which control the transformation of petunidin to malvidin, were in a recessive state, there was synthesis of a small quantity of malvidin which was strongly correlated (r = 0.96) with the amount of petunidin synthesized.

3 *Quantitative Estimation of Different Anthocyanins Synthesized*

The relative accumulation of delphinidin in the light-coloured types prompted an analysis of the different anthocyanins. It was thus possible to differentiate delphinidin-3-glucoside and delphinidin-3-rutinoside from the other substituted 3-5 pigments (delphinidin, petunidin, malvidin-3 p-coumaroyl-rutinosido-5-glucoside). The relative quantity of each pigment, expressed as a percentage of the total pigment content, in each extract is shown in Table 2. In spite of the presence of the *Rt* gene (in the heterozygous state) which allows the transformation of delphinidin-3-glucoside to delphinidin-3-rutinoside and that of the *Gf* gene (in the homozygous state) which controls the synthesis of substituted acylated 3-5 pigments, many of the variants still accumulated 3 substituted forms of delphinidin (Dp-3G and Dp-3RG). The light-coloured types accumulated notable quantities of Dp-3RG and Dp-3G in the proportion of 3 to 1. They were very clearly differentiated from the recessive control in which 50% of the pigments consisted of Dp-3G. As the quantity of total pigments increased, Dp-3G then Dp-3RG tended to disappear giving place to substituted 3-5 types. There seemed to be a threshold effect for both (0.170 for Dp-3G and 0.250 for Dp-3RG) which sharply defined firstly a total efficiency of the *Rt* gene followed by that of the *Gf* gene.

The substituted pigment consisted largely of petunidin-3 p-coumaroyl-rutinosido-5 glucoside. However, in the dark-coloured types there was always a delphinidin present in fairly constant amounts amongst the aglycones. This delphinidin was, like petunidin, a substituted acylated 3-5 pigment. This shows that even amongst the darkest types total efficiency of the methylation genes *Mt1* and *Mt2* (Table 1) does not occur.

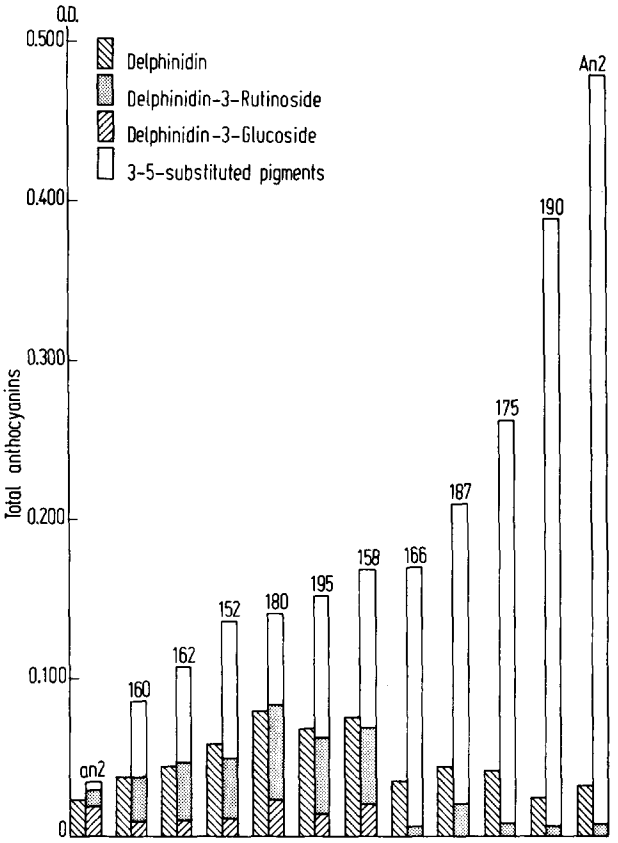

Fig. 2. Anthocyanin synthesis in *an2-n* variants

Discussion

For the experimental conditions and the genetic background reported in the present paper, the heterozygotes *An2 an2* have, in quantitative terms, an anthocyanin synthesizing ability which is about fifteen fold greater than that of the recessive homozygote *an2 an2* (Table 2). The synthesizing abilities of the stable variants lie between those of these two control plants. Since all the variants had heterozygous formulas including *an2*, the anthocyanin content of the lightest ones, visually selected against the recessive phenotype, was obviously greater than that of the homozygous recessive plants. There was, on the other hand, no selection limit for the darker types, which means that in the present experiments variants reaching the *An2 an2* level must not have occurred. It can therefore be concluded, at least for the germinal tissue, that the functioning of the unstable system *an2-1* does not usually return to that of the fully functioning *An2* gene. This is

not generally the case in *Petunia* since phenotypically assessed reversions towards wild type can be observed using the unstable system *an2-14* (Cornu, 1977) or unstable mutants at *An1* locus (Bianchi 1978).

Following Barbara McClintock's studies on maize (1965) several hypothesis have been proposed to explain the genetic instabilities. The most recent supposed an integration of a segment of DNA, analogous to insertion segment behaviour in bacteria, into the locus which then becomes unstable (Peterson 1970, Nevers et Saedler 1977). This inserted segment of DNA, suggested as being a part of or a complete controlling element, could cause either reduction or a total block in transcription of the gene concerned. The DNA segment could then be transposed or removed by excision. According to this hypothesis, in *Petunia* the unstable mutation *an2-1* would be the consequence of the integration of an insertion segment into the *An2* locus, resulting in a drastic decrease in gene expression. The occasional excision of this insertion segment would give rise to the new *an2-n* stable alleles, derived from the *An2* gene and would be responsible for pigment variegation in the flowers. In the present experiments these new derivatives did not reach the functional level of the original *An2* gene, possibly, as occurs in bacteria and maize, because of inaccurate excisions leading to different extents of intragenic deletion (Nevers and Saedler 1977, Reddy and Paterson 1976). It is well established in *Petunia* that deletion induces a low transmission level of deleted male gametes. This hypothesis would therefore explain the inequality in transmission levels of the *an2-n* alleles as compared to those of the *an2* allele. Even though female gametes give a standard transmission, transmission of the *an2-n* allele, as compared to the *an2* allele, by the male gametes is regularly depressed (Table 3, Cornu unpublished data). This may result from an inaccurate excision of the insertion element leading to loss of DNA. For the *An2* locus, however, it should be emphasized that there is no evidence available for the occurrence of an external controlling element regulating the excision or the transposition of the internal element.

Although there is no qualitative difference in the types of anthocyanins produced either by the *an2 an2*, the *an2-n an2* or by the *An2 an2* plants, their ratios vary. The amount of the 3-substituted pigments, quite important in the light coloured plants is very small in the darker ones (Table 2). The variations occurring by gradations express the increasing efficiency that acts sequentially upon the successive genes controlling the anthocyanin pathway. Transformation of delphinidin 3-glucoside to delphinidin 3-rutinoside seems to be a function of the amount of precursor available. It reaches about 100 per cent in *an2-166* plants where the total anthocyanins are at the .169 level (Table 2). The same is true for the 5-substituted pigments whose synthesis is virtually total at the .255 level. Since the amount of available precursor seems to be related to the nature of the *an2-n* allele, it could be that its synthesis is controlled by the *An2* locus. In this case, the regulatory like effect of the latter on the last steps of anthocyanin biosynthesis may be either: 1) by influencing the transcriptional activity of the gene *Rt*, this then regulating the activity of the gene *Gf*, etc. ..., by means of a sequential gene regulation as in the model of Britten and Davidson (1969) or 2) by induction of the enzymes in function of the amount of each successive substrate produced (Dooner and Nelson 1977).

A study of the kinetics of anthocyanin biosynthesis in plants with different *an2-n* alleles would probably enable a better understanding of the interaction between the genes concerned. Moreover, since the enzyme UDP glucose: anthocyanidin 3-0-glucosyl transferase is controlled by the gene *An2* (Kho and al 1978), an investigation of the activity, and eventually the quantity, of this enzyme could lead to a better insight into the functioning of the gene *An2* and the nature of the *an2-n* alleles themselves.

Table 3. Frequencies of transmission of *an2-n* alleles versus *an2* allele in reciprocal testcrosses between variants and recessive tester

Allele *an2-n*	Transmission frequencies (%) when variant is ♀	variant is ♂
152	48.2	31.0[a]
157	50.9	35.7[a]
160	50.9	14.2[a]
171	48.2	47.1
173	46.8	22.4[a]
176	48.3	6.3[a]
182	46.9	3.5[a]
189	51.8	10.5[a]

[a] Frequency significantly ($P(\chi^2) < .05$) different from 50%

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