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The genetic control of the enzyme UDP-glucose: 3-0-flavonoïd-glucosyltransferase in flowers of *Petunia hybrida*

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Summary. Four genes controlling the conversion of dihydroflavonols into anthocyanins have been investigated for their effect on UDP-Glucose: 3-0-flavonoïd glucosyltransferase activity, one of the enzymes involved in this conversion. *An1* and *An2* control the bulk of UFGT activity; a homozygous recessive for one of these genes shows an activity of 5–20% of the wildtype value.

In a homozygous double recessive some 5% activity is still found while in mutants homozygous recessive for *An6* or *An9*, UFGT activity is lower. In F2 progenies segregating for *An6* or *An9*, however, no difference in UFGT activity was found between homozygous recessive and dominant plants.

Mutants blocked in a biosynthesis step preceding the formation of dihydroflavonols show normal UFGT activity levels, indicating that no anthocyanidins are needed for UFGT induction. In addition to delphinidin, myricetin was used as a substrate. The results obtained indicate the probability that both substrates can be glucosylated by the same UFGT enzyme.

Key words: Flavonoïds – Glucosyltransferase – Genetic control – *Petunia hybrida*

Introduction

The conversion of dihydroflavonols into anthocyanins involves the glucosylation of the 3-hydroxy position of the molecule. This reaction is catalyzed by the enzyme UDP-Glucose: 3-0-flavonoïd glucosyltransferase (UFGT).

The enzyme activity has been described for *Zea mays* (Larson and Coe 1968; Dooner 1981), *Brassica oleracea* (Saleh et al. 1976), *Silene dioica* (Kamsteeg et al. 1978) and *Tulipa* cultivars (Wierman et al. 1980). In *Petunia hybrida* the enzyme activity was first described by Kho et al. (1978); it requires UDPG as a glucose donor and glucosylates both delphinidin and cyanidin aglucons. In preliminary experiments Kho et al. (1978) showed that in flowers of *Petunia hybrida* UFGT activity is controlled by the genes *An1* and *An2*.

Unstable alleles have been described for the genes *An1* and *An2* (Bianchi et al. 1978; Cornu 1977 respectively). Derivative alleles of unstable systems of both genes can be ranked in an order of increasing anthocyanin synthesis between the normal recessive and dominant phenotypes (Farcy and Cornu 1979; Gerats et al., submitted).

Recently, two more genes (*An6* and *An9*) involved in the conversion of dihydroflavonols into anthocyanins have been described (Gerats et al. 1982 b).

These observations prompted us to study the genetic control of UFGT activity.

Materials and methods

Plant material

All plants were grown in the greenhouse. Flowerbuds containing a maximal UFGT activity (Kho et al. 1978), were used for enzyme extraction.

Enzyme extraction

Two methods of enzyme extraction were used. Method 1, using polyclar AT, required 5–10 flowerbuds, which were homogenized with a Potter homogenizer in 3 ml extraction buffer (50 mM potassium phosphate, pH 7.5, 5% PVP (w/V), 20 mM β-mercaptoethanol). The homogenate was centrifuged at 40,000 g for 20 min after which the supernatant was filtered through a paper filter and poured onto a polyclar AT column (10×0,5 cm), equilibrated with 10 mM potassium phosphate (pH 7.5; 4 mM β-mercaptoethanol). Fractions containing the bulk of protein were pooled and used in assays. Method 2 required two flowerbuds, which were homogenized with pestle

and mortar. The following additions were made to the buds: a little quartz sand, an excess of Dowex 1×2 and 1 ml buffer (30 mM potassium phosphate, pH 6.5; 20 mM β-mercaptoethanol).

The homogenate was centrifuged at 40,000 g for 10 min. The resulting supernatant was used in assays.

In both methods all steps were performed at 0–4 °C.

Enzyme assays

Fifty to ninety µl enzyme extract was allowed to react for 30–120 s at 30 °C in a reaction mixture containing 10 mM phosphate buffer (pH 7.5), 1 mM UDPG, 20 µM delphinidin (in 5 mM HCl) and H_2O in a total volume of 200 µl. The reaction was started by the addition of delphinidin and terminated by the addition of 800 µl chloroform : methanol 2 : 1 (0.5% HCl), resulting in a Folch partition (Folch et al. 1957). Anthocyanins concentrated in the upper phase (400 µl). Part of the upper phase was injected into a series 3B (Perkin and Elmer) liquid chromatograph equipped with a Lichrosorb 10RP18 reverse phase column (24×0.5 cm). Detection occurred with a LC75 (Perkin and Elmer) variable wavelength detector at 530 nm.

Anthocyanins were eluted in a 7 min gradient of 20% to 50% methanol in a 10% formic acid solution at a flow rate of 4 ml/min and a temperature of 45 °C. Under these conditions the retention times for delphinidin-3-glucoside and delphinidin are 2.9 min and 4.7 min respectively.

The myricetin assay contained the following components: Tris HCl (10 mM, pH 9.0), UDPG (1 mM), myricetin (50 µM, in EGME) and 100 µl enzyme extract in a total volume of 200 µl. The reaction was started by the addition of myricetin and subsequently stopped by the addition of 800 µl chloroform : methanol 2 : 1. Detection occurred at 350 nm after separation at 30 °C in a 7 min gradient of 33 to 66.5% methanol in a 5% HAc solution.

Glucose-6-phosphate dehydrogenase (G6PD) activity was measured spectrophotometrically. The assay contained Tris HCl (127 mM, pH 8.0), $MgCl_2$ (6.6 mM) glucose-6-phosphate (2 mM), H_2O, 50 µl enzyme extract and NADP (0.75 mM) (not in the blanco). The reaction was started by the addition of NADP and the increase in absorption at 340 nm was followed for 5 min.

α-D-mannosidase activity was measured spectrophotometrically. The assay contained 25 µl sodium acetate buffer (125 mM, pH 4.0), 50 µl p-nitrophenyl-α-D-mannopyranoside (0.83 mM in H_2O), 50 µl enzyme extract and H_2O up to a total volume of 200 µl.

The reaction was started by the addition of the enzyme extract and was terminated after 10 min at 30 °C by the addition of 800 µl glycine-NaOH buffer (1 M glycine, pH 10.6). The extinction at 405 nm was measured within 2 h after the reaction was carried out. G6PD activity was measured in polyclar AT extracts as a reference enzyme; α-D-mannosidase activity was likewise measured in Dowex extracts.

Protein was determined using the Biorad Protein Assay (Bradford 1976), with Bovin serum albumin as a standard protein.

Results and discussion

Coloured flowering mutants and mutants homozygous recessive for any one of the genes *An1, An2, An3, An6* or *An9* were investigated for their UFGT activity in flowerbuds approaching maturity. Furthermore UFGT activity was measured in mutants homozygous recessive for both *An1* and *An2, An2* and *An3* or *An2* and *An9* (Table 1). UFGT activity was expressed as Specific Activity (SA) or as a ratio over the activity of a non-flavonoïd enzyme. The ratio was used as an expression of activity, independent of the result of protein extraction and determination. In method-1-extracts (see "Materials and methods"), G6PD activity was used as a reference. In method-2-extracts, we used α-mannosidase activity as the reference because G6PD activity in these extracts appeared to be almost zero. To find out whether flavonols and anthocyanidins are glucosylated at the 3-place by the same enzyme, myrecitin and delphinidin were used as substrates. The results are presented in Table 2.

Table 1. Lines, used in the present investigation

Coloured	R3, R27, R78, R93, R118, R127 M1, M43, M57, M61, M73 V28, V32, V46
an1an1	W20, W24, W42, W45, W46, W78
an2an2	W15, W22, W60, W71, W95
an3an3	W37, W39, W67
an6an6	W79, W80, W89, W91, W102
an9an9	W75, W84, W87, W97, W98, W99
an1an1 an2an2	W121, W126, W127
an2an2 an3an3	W130
an2an2 an9an9	W125, W132

In mutants homozygous recessive for *An1* or *An2*, UFGT activity is reduced as compared to coloured mutants. A residual activity of 5–20% was found. This result confirms the observations of Kho et al. (1978). Although *An1* and *An2* both control 80–95% of the UFGT activity, a mutant homozygous recessive for both genes still shows a considerable residual activity (Table 2). In *Zea mays* a very similar situation is found for several enzymes (Hannah et al. 1980; Chourey 1981).

Either there exists a third gene controlling a UFGT activity or there is a closely related enzyme (for example a 5-glucosyltransferase) which exhibits affinity for glucosylation at the 3 hydroxy group.

UFGT activity in *an3an3* mutants is comparable to that of coloured lines. These mutants are blocked in an early step of flavonoïd biosynthesis and are white flowering or synthesize only minor amounts of anthocyanins. A possible regulation of gene expression by anthocyanidin can therefore be excluded. Mutants homozygous recessive for *An6* or *An9* exhibit a lowered UFGT activity as compared with activity in coloured mutants. The controlling effect of *An1, An2, An6* and

Table 2. UFGT activity in a series of mutants of *Petunia hybrida*

Genotype for[f] *An* genes	Method 1 extract[a]				Method 2 extract[a]	
	del[b] as substrate		myr[c] as substrate		del[b] as substrate	
1 2 3 6 9	SA[d]	Ratio[e]	SA[d]	Ratio[e]	SA[d]	Ratio[e]
+ + + + +	43.3±32.6 (*n*=12)	19.3±12.4	26.2±17.4 (*n*=7)	12.1±8.2	64.4±13.4 (*n*=12)	977 ±221
− + + + +	5.2± 2.2 (*n*=7)	2.6± 1.0	5.8± 3.3 (*n*=6)	2.4±1.3	5.4± 3.1 (*n*=6)	80.7± 34
+ − + + +	7.9± 2.5 (*n*=5)	3.6± 1.8	6.6± 4.5 (*n*=5)	2.6±1.2	3.3± 1.5 (*n*=5)	51.6± 13
+ + − + +	39.5± 8.5 (*n*=5)	14.9± 3.7	30.5±10.3 (*n*=5)	11.3±3.3	77.0 (*n*=5)	946
+ + + − +	30.1±17.8 (*n*=8)	10.9± 6.7	23.6± 9.9 (*n*=7)	8.6±3.9	44.0 (*n*=2)	760
+ + + + −	18.6± 8.5 (*n*=9)	6.8± 3.3	23.1±12.7 (*n*=7)	7.5±3.4	13.4 (*n*=2)	200
− − + + +	2.4± 2.8 (*n*=7)	1.0± 1.0				
+ − − + +	5.4 (*n*=2)	1.9				
+ − + + −	12.7± 4.5 (*n*=3)	4.0± 3.0				

[a] See M+M; [b] del = delphinidin; [c] myr = myricetin; [d] Specific activity in mU/min; [e] ratio: SA UFGT ref enzyme, G6PD for method 1 extracts, α mann for method 2 extracts; [f] for the lines used, see Table 1

Table 3. UFGT activity in parental lines and F2 progenies, segregating for *An1, An2, An6* or *An9*

Phenotype	Genotype	SA UFGT[a]	Ratio[b]	*n*[c]
R27	*An An*[d]	52.8± 3.6[e]	982±141[e]	3
W78	*an1an1*	3.7± 0.6	62± 11	3
F2, coloured	*An1-*	44.1± 9.8	815±232	23
F2, colourless	*an1an1*	8.6± 3.0	160± 59	3
W15	*an2an2*	1.8	50	2
F2, coloured	*An2-*	41.1±12.0	780±229	31
F2, colourless	*an2an2*	5.9± 2.6	84± 35	7
W89	*an6an6*	48.3	690	1
F2, coloured	*An6-*	44.8±12.5	–	3
F2, colourless	*an6an6*	42.4± 5.7	–	3
W75	*an9an9*	31.1[f]	–	1
F2, coloured	*An9-*	28.9± 3.2	–	3
F2, colourless	*an9an9*	32.5± 8.7	–	3

[a] SA in mU/min (method-2-extracts); [b] ratio: SA UFGT/SA α mannosidase; [c] *n*: number of plants tested; [d] R27 was used as a dominant tester in all four crosses; [e] mean ± SD; [f] act measured in a method-1-extract

An9 on UFGT activity was further investigated in the progeny of F2 crosses (Table 3). In the F2 progenies of the crosses W89×R27 (*an6an6*×*An6An6*) and W75×R27 (*an9an9*×*An9An9*) the white flowering plants had a UFGT activity equal to that of the coloured progenies. This indicates that neither *An6* nor *An9* is directly involved in the control of UFGT activity. In the crosses W78×R27 (*an1an1*×*An1An1*) and W15×R27 (*an2an2*×*An2An2*) the white flowering progenies showed decreased UFGT activities. From this we conclude that UFGT activity in *Petunia hybrida* is controlled by the genes *An1* and *An2*.

The results obtained by using myricetin as a substrate parallel those obtained with delphinidin as a substrate (Table 2). This indicates that the same enzyme is responsible for glucosylation of the 3-hydroxy group of both delphinidin and myricetin. This is confirmed by the occurrence of co-chromatography of both activities under a variety of conditions (L.M.V. Jonsson, personal communication). It should be mentioned that activity measurements as presented in Tables 2 and 3 were performed using extracts from flowerbuds approaching maturity. The effect of *An1* and *An2* on UFGT activity during flower development is shown in Fig. 1. In a coloured line and in an *an3an3* mutant, UFGT activity increased with increasing bud length. In the lines W78 (*an1an1*) and W15 (*an2an2*) there was no increase in UFGT activity during flower development. Either there is no transcription or transcription leading to a non-functional enzyme, or the messenger or enzyme is degraded rapidly.

In young buds of *an1an1* and *an2an2* mutants UFGT activity was relatively high. Anthocyanin synthesis itself, however, was restricted to later stages of flower development (Gerats et al. 1982a). Kho et al. (1978) suggested that UFGT is a dimeric enzyme. One can hypothesize that both *An1* and *An2* contribute to

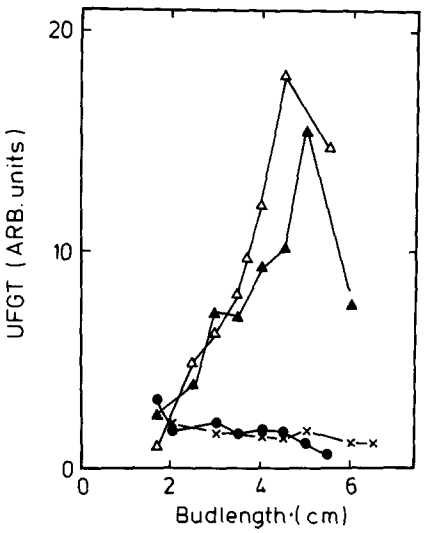

Fig. 1. Glucosyltransferase activity during flower bud development. Extracts were made using method 2 (see "Materials and methods"). △—△ W39 (*an3an3*), ●—● W15 (*an2an2*), ▲—▲ M43 (*An An*), ×—× W78 (*anlanl*)

the structure of the enzyme, the enzyme thus being a heterodimer. Another possibility is that one or both genes act as a regulating gene for UFGT.

It is known that both *An1* and *An2* show pleiotropic effects. *An1* controls the expression of the gene *Hf1* as well (Tabak 1981; Gerats et al. 1982b), whereas *An2* controls the expression of genes involved in the modification of the anthocyanin molecule (Farcy and Cornu 1979).

Further research on the effect of *An1* and *An2* on UFGT and other processes controlling anthocyanin synthesis could provide more information. The allelic series of *An1* and *An2* will especially be of great interest.

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