Common Identity of UDP-Glucose: Anthocyanidin 3-O-Glucosyltransferase and UDP-Glucose: Flavonol 3-O-Glucosyltransferase in Flowers of *Petunia hybrida*

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In an attempt to distinguish between the UDP-glucose: flavonol 3-O-glucosyltransferase (3GT) and the UDP-glucose: anthocyanidin 3-O-glucosyltransferase in flower buds of *Petunia hybrida*, several properties of these activities were determined. The 3-glucosylation of anthocyanidin had a pH-activity optimum of 7.2, that of flavonol pH 9.2 to 9.5. Anthocyanidin 3GT activity was lowered in the presence of EDTA or β-mercaptoethanol, but this was due to an effect on the anthocyanidin substrate. The two 3-glucosylating activities were to a similar extent inhibited by an increasing ionic strength in the enzyme assay and showed an identical iso-electric point (5.2) as determined by chromatofocusing. Molecular weights were identical: 26000, 52000 or 78000 daltons as determined by gel-filtration. Antiserum raised against partially purified 3GT gave identical immunoprecipitation curves with flavonol 3GT and anthocyanidin 3GT.

Special attention was given to the 3-O-glucosyltransferase in mutants with low levels of 3GT activity. These mutants are unable to form significant amounts of anthocyanins but contain wild-type amounts of flavonols. The enzyme of such mutants had the same iso-electric point and identical titration-curves with antiserum as the enzyme from wildtype plants. 3GT from wildtype or mutant plants glucosylated flavonols at higher rates than anthocyanidins.

Introduction

The first anthocyanidin-modifying enzyme ever described was the UDP-glucose: anthocyanidin 3-O-glucosyltransferase [1, 2]. This enzyme catalyzes the glucosylation at the 3-hydroxy position of the anthocyanidin molecule. Anthocyanins lacking a glucose at this position are utterly rare. Studies of the anthocyanidin 3-O-glucosyltransferase (3GT) in several species have since then been reported. Saleh and co-workers used cell cultures of *Haplopappus gracilis* as source of enzyme, and subsequent enzymic studies (reviewed in [3]) were carried out using various plant material: cabbage seedlings [4], flowers of *Silene dioica* [5] and flowers from *Petunia hybrida* [6].

Abbreviations: EDTA, ethylene diamine tetraacetic acid; EGME, ethylene glycol monomethyl ether; HPLC, high performance liquid chromatography; 3GT, 3-O-glucosyltransferase.

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As yet, it is not certain whether the anthocyanidin 3GT is also capable of glucosylating flavonols at the 3-OH position. The partially purified enzymes from *Haplopappus* and *Brassica* glucosylated flavonols at high rates [2, 4]. Likewise, the flavonoid 3GT from maize pollen, extensively used in genetic studies [7] is supposed to glucosylate anthocyanidins as well as flavonols [8]. In contrast, neither could the anthocyanidin 3GT from *Silene* glucosylate flavonols [5], nor did the flavonol 3GTs from soybean cell cultures [9], *Pisum sativum* [10] or Tulip anthers [11] glucosylate anthocyanidins at significant rates.

In flowers of *Petunia hybrida*, anthocyanidin 3-glucosides as well as flavonol 3-glucosides are synthesized, as is illustrated in Fig. 1. Accordingly, studies *in vitro*, using crude enzyme preparations, revealed the occurrence of both anthocyanidin- and flavonol 3-glucosylating activities. When the genetic control of the 3GT in different mutants was investigated, a correlation between the two activities was found: mutants showing low levels of anthocyanidin 3GT activity also had a low flavonol 3GT activity [12]. This suggests that we are dealing with one and

the same enzyme. In contrast with these findings is the difference in accumulation of anthocyanins and flavonols in some particular mutants. Plants homozygous recessive for one of the two genes *An1* or *An2* show only about 5–20% of the normal glucosyltransferase activity [6, 12]. These plants are either white-flowering (*an1an1*-mutants) or they contain small amounts of anthocyanin, giving a weakly coloured flower (*an2an2*-mutants). This indicates a correlation between the 3GT activity and the amount of 3-glucosylated product. On the other hand, *an1an1*- and *an2an2*-mutants contain normal levels of the flavonols kaempferol and quercetin [13]. The flavonols occur mainly as 3-diglycosides ([14], P. de Vlaming, unpublished results) which suggests that the residual 3GT activity in these mutants is different from the one in normal plants, supposedly a flavonol-specific 3GT.

In order to find out whether one and the same enzyme is responsible for 3-glucosylation of anthocyanidins and flavonols in flowers of *Petunia hybrida*, several properties of the two 3GT activities were determined in wildtype and mutant (*an1an1* or *an2an2*) lines. Furthermore, antiserum was raised against partially purified 3GT and tested on the capacity to precipitate the flavonol 3GT and the anthocyanidin 3GT, respectively.

The results of these studies, which all support the idea of a common identity of flavonol 3GT and anthocyanidin 3GT in *Petunia hybrida*, are reported in this paper.

Materials and Methods

Plant material

All plants (*Petunia hybrida* hort.) were grown in a greenhouse. The following inbred lines were used: R27, V13 (*Fl*-), W22 (*an2an2*), W42 (*an1an1*) (see [15] for a description of the genes).

Enzyme extraction

All steps were performed at 0–4 °C. Limbs of flowers and/or flower buds were homogenized with buffer I (see below for buffer compositions) and some quartz sand in a mortar. During homogenization, Dowex 1X2-200 (Sigma, St. Louis, USA) at half the amount of the fresh weight of the tissue was added. The homogenate was centrifuged at 38000 *g* for 20 min. The supernatant was chromatographed on a Sephadex G-25 column (1.5 × 8.3 cm) which had been pre-equilibrated with buffer VI. The same buffer was used for elution, and fractions containing protein were pooled for assay of 3-O-glucosyltransferase (3GT) activity and protein content. Protein was determined according to Bradford [16] with bovine serum albumin as standard protein.

Molecular weight determination

Molecular weights were determined by gel-filtration of a Sephadex G-25 eluate essentially prepared as described in the preceding section. Samples were prepared from the red-flowering mutant R27 and applied to a calibrated Sephacryl S-200 Superfine (Pharmacia, Uppsala, Sweden) column (1.6 × 60 cm), which was eluted at 35 ml/h. Fractions of 1 ml were collected. The following variations were applied during extraction of enzyme:

I. 48 limbs of flower-buds (2.5 g fresh weight) were homogenized in 16 ml of buffer V.
II. 56 limbs of flower-buds (3.3 g fresh weight) were homogenized in 10 ml of buffer IV.
III. 48 limbs of flower-buds (2.5 g fresh weight) were homogenized in 16 ml of buffer III.

In all experiments a 2.5 ml aliquot of the supernatant after centrifugation at 38000 *g* during 20 min was chromatographed, first on a Sephadex G-25 column and then on a Sephacryl S-200 Superfine column. The chromatographed enzyme samples contained the following amounts of protein: I: 3.6 mg, II: 12.0 mg, III: 3.7 mg.

Chromatofocusing of enzyme

Chromatofocusing experiments were carried out as described before [17], but without buffering of collected fractions.

Partial purification of 3-O-glucosyltransferase and preparation of antiserum

The purification and immunization procedures have been described elsewhere [17].

Incubation of enzyme with anti-3GT serum immobilized to Protein A-Sepharose CL-4B

The coupling of anti-3GT to Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was carried out at 20 °C during 2 h, with gentle rotation in

Eppendorf tubes. To each tube, containing 7.5 mg Protein A-Sepharose CL-4B, 0.1, 0.2, 0.4, 0.8 or 1.6 mg anti-3GT serum protein in 250 µl phosphate-buffered saline (pH 7.4) were added. Control incubations contained corresponding amounts of normal rabbit serum protein. The gel was washed three times with phosphate-buffered saline (pH 7.4) by mixing on a Vortex and centrifugation (10 s, 10000 x*g*), and thereafter incubated with enzyme sample. After 1 h incubation at 20 °C with gentle rotation, the gel was centrifuged (10 s, 10000 x*g*) and the enzyme activities in the supernatant were determined.

The following enzyme preparations were used in the incubations:

W42 (*an1an1*): A crude enzyme preparation after elution on a Sephadex G-25 column with buffer II. Each sample contained 420 µg protein in a volume of 100 µl (123 pkat quercetin 3GT activity).

V13 (wildtype) and W22 (*an2an2*): A partially purified 3GT-preparation, obtained after chromatofocusing. The samples contained 28 µg protein, 143 pkat quercetin 3GT activity (V13) or 6 µg protein, 123 pkat quercetin 3GT activity (W22) in 100 µl buffer II.

Glucosyltransferase assay

Standard glucosyltransferase assays were performed as described before [12]. Differing conditions are described in the Results section. Quantitative analysis of the glucosylated products, using high performance liquid chromatography (HPLC), was performed using a Series 3B (Perkin and Elmer) liquid chromatograph equipped with a Lichrosorb 10RP18 reverse phase column (24 × 0.5 cm). Elution was carried out with a solution of 10% formic acid in water (v/v) with varying amounts of methanol. The elution programs were adjusted to obtain a separation of glucosylated product and aglucone substrate within 4 min, as shown in Table I. The flow rate was 4 ml/min at 45 °C. An LC 75 (Perkin and Elmer) variable wavelength detector was used at 530 nm (anthocyanins) or 350 nm (flavonols). A mM extinction coefficient of 34 in methanol-0.5% HCl (v/v) was applied to anthocyanins.

Buffer solutions

The following potassium phosphate buffer solutions were used: (I) 100 mM, pH 8.5, containing 1.4 mM β-mercaptoethanol, (II) 100 mM, pH 7.4, (III) 50 mM, pH 7.5, containing 20 mM β-mercaptoethanol, (IV) 50 mM, pH 8.5, containing 20 mM β-mercaptoethanol, (V) 50 mM, pH 7.0, containing 25 mM β-mercaptoethanol, (VI) 10 mM, pH 7.5.

Results and Discussion

a. Effects of extraction procedures and incubation mixture on anthocyanidin 3GT activity and flavonol 3GT activity in vitro

Analysis of substrate and product

In earlier work on the anthocyanidin 3GT, the activity was measured using [UDP^{14}C]glucose as glucose donor and recording the amount of radioactivity incorporated in the 3-glucosylated product, after separation using paper chromatography [2, 4–6]. We used HPLC to obtain a rapid separation of product and substrate, which was advantageous in view of the low stability of anthocyanin compounds. A second advantage was that the method allows a determination of the recovery of substrate after incubation. This is illustrated in the first section, describing effects of extraction procedure and incubation mixture on the activity of the two 3GTs *in vitro*.

Table I. Elution programs used for HPLC-analysis.

Substrate	% Methanol	Gradient (min)	Equilibration (min)	Retention time (min)	
				Aglucone	3-glucoside
Delphinidin	17.5 to 25%	2.5	1.5	2.80	1.55
Cyanidin	20 to 27.5%	2.5	1.5	3.50	1.80
Kaempferol	36%	isocratic		3.25	2.00
Quercetin	25%	isocratic		3.50	1.85
Myricetin	15%	isocratic		4.00	2.60

Table II. Effect of β-mercaptoethanol on 3GT activities.

Conc. βmOH mM	Recovery of substrate		Activity (nkat/mg protein)			
	Delphinidin	Quercetin	Delphinidin 3GT		Quercetin 3GT	
0	100%	100%	1.76	100%	3.27	100%
0.3	90%	100%	1.60	91%	3.28	101%
1.1	80%	100%	1.44	82%	3.27	100%
4.0	40%	100%	1.30	75%	3.33	102%

βmOH = β-mercaptoethanol
Enzyme assays were performed for 3 min. The anthocyanidin assay contained 15 mM potassium phosphate pH 7.5, 80 μM delphinidin, 1% EGME (v/v) and 11 μg protein. The flavonol assay contained 200 mM Tris-potassium phosphate pH 9.5, 200 μM quercetin, 20% EGME (v/v) and 11 μg protein.

Effects of β-mercaptoethanol

Crude enzyme samples were prepared by extraction in a buffer containing β-mercaptoethanol, followed by centrifugation and chromatography on a Sephadex G-25 column, using an elution buffer without SH-reagent. When extraction was performed without β-mercaptoethanol in the buffer, both anthocyanidin 3GT and flavonol 3GT activities were lowered, but to various degrees (46% and 75% of control, respectively). This effect appeared to be due to the presence of a flavonoid-degrading component in extracts prepared without SH-reagent. The component eluted in the void volume of a Sephadex G-25 column and thus has to be a protein, possibly a phenol-oxidase [18]. A 1.4 mM concentration of β-mercaptoethanol in the extraction buffer was sufficient to inactivate the flavonoid-degrading enzyme.

After chromatography on a Sephadex G-25 column, the enzyme preparation did not contain β-mercaptoethanol. If this compound then was added during enzyme incubations, the activity of anthocyanidin 3GT was strongly affected, whereas the flavonol 3GT was not influenced, as is shown in Table II. The difference was due to the degradation of anthocyanidin in the presence of β-mercaptoethanol, which is also illustrated in Table II. The flavonol substrate was not appreciably affected.

Effect of ethylene diamine tetraacetic acid (EDTA)

Kho and co-workers [6] reported that EDTA reduced product formation caused by anthocyanidin 3GT. In our experiments, these results were confirmed. It appeared that the anthocyanidin substrate decomposed in the presence of EDTA. After incubation during 2 min in an assay with 100 μM EDTA, only 60% of the anthocyanidin was present, as compared to a control incubation without EDTA. An equal concentration of EDTA did neither influence the flavonol substrate nor the flavonol 3GT activity. Adding EDTA to the extraction buffer did not affect the 3GT, whether measured with flavonol or anthocyanidin substrate.

Inhibition at higher ionic strength in the assay

The ionic strength had a strong influence on both 3GT activities. This is illustrated in Table III. The effect was observed at different pH-values and with different buffer solutions: delphinidin 3GT at pH 7.5 (potassium phosphate) and pH 7.2 (2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-potassium phosphate); quercetin 3GT at pH 8.0 (Tris-HCl) and pH 9.5 (Tris-potassium phosphate).

Table III. Effect of ionic strength on 3GT activities.

Conc. of buffer mM	Activity (nkat/mg protein)			
	Delphinidin 3GT		Quercetin 3GT	
10	1.43	100%	5.20	100%
50	1.12	78%	4.59	88%
100	0.88	62%	3.75	72%
150	0.87	61%	3.42	66%

Assays were incubated for 2 min. The flavonol assays contained Tris-potassium phosphate buffer pH 9.5, 200 μM quercetin, 20% EGME (v/v) and 11 μg protein. The anthocyanidin assays contained potassium phosphate buffer pH 7.5, 80 μM delphinidin, 1% EGME (v/v) and 11 μg protein.

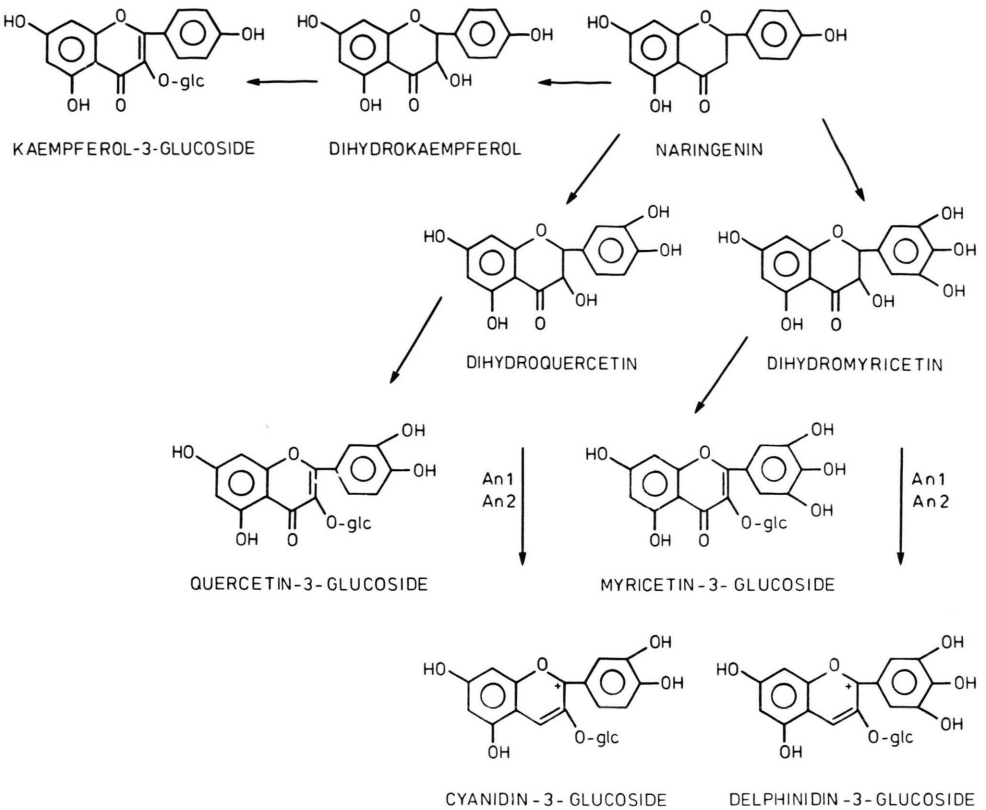

Fig. 1. Part of the biosynthetic pathway of anthocyanins and flavonols in flowers of *Petunia hybrida*. When one of the genes *An1* or *An2* is homozygous recessive, no anthocyanins are found. Redrawn from [13]. The flavonols mainly accumulate as 3-diglycosides.

pH-activity profiles

The pH-activity profiles of the 3GTs are shown in Figure 2. Delphinidin 3GT had a pH-activity optimum at pH 7.2. At pH-values above 7.7, the anthocyanidin was rapidly degraded. Quercetin 3GT showed the highest activity at pH 9.2 with glycine-NaOH buffer, and at pH 9.5 with a buffer containing Tris and potassium phosphate. Other flavonol 3GTs had pH-activity optima around pH 8.0 [9, 10, 19, 20]. It should be noted that the *Petunia* flavonol 3GT, like the flavonol 3GT isolated from *Haplopappus* [20] was two times as active in glycine-NaOH buffer as in Tris-buffer (pH 9.0).

The difference in pH-activity optima of the two 3GT-activities does not provide evidence that there exist two 3GT-enzymes, since the two substrates have different chemical properties, affecting the activity *in vitro*. The pH-activity optimum of the flavonol 3GT seems too high to be of significance *in vivo*.

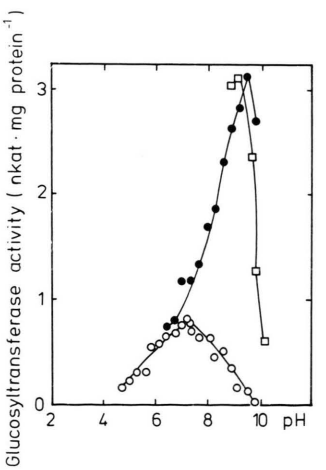

Fig. 2. Effect of pH on delphinidin 3GT and quercetin 3GT. The anthocyanidin incubation (○—○) contained 80 µM delphinidin, 1% EGME (v/v) and 200 mM Tris-potassium phosphate. The flavonol incubations contained 200 µM quercetin, 20% EGME (v/v) and 200 mM Tris-potassium phosphate (●—●) or 200 mM glycine-NaOH (□—□). The activities of the incubations with glycine-NaOH are given at 50% of the measured value.

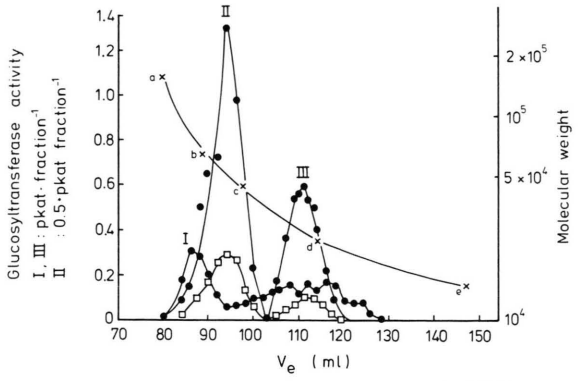

Fig. 3. Molecular weight determination of 3GT on a Sephacryl S-200 Superfine column. a = aldolase, b = bovine serum albumin, c = ovalbumin, d = trypsinogen, e = lysozyme. (●—●) = quercetin 3GT, (□—□) = delphinidin 3GT. The enzyme samples were prepared as described in the Methods section. Incubations with quercetin contained 150 mM glycine-NaOH pH 9.2; with delphinidin 15 mM potassium phosphate pH 7.4. Anthocyanidin 3GT activity in experiment I was very low, and is therefore omitted.

b. Physicochemical properties of the anthocyanidin 3GT activity and the flavonol 3GT activity

Molecular weight

The molecular weights of the 3GT activities were determined by gel-filtration. In Fig. 3 the results of these experiments are illustrated. Under different conditions of extraction, the delphinidin 3GT and the quercetin 3GT showed identical apparent molecular weights. These molecular weights varied, depending on pH, ionic strength and protein concentration during extraction. In experiment III (50 mM potassium phosphate buffer pH 7.5 during extraction), 3GT activity was found only in fractions corresponding to a M_r of 26000. A more concentrated homogenate, extracted with a buffer of higher pH (pH 8.5), gave a molecular weight of 52000 (II). Finally, when extraction was carried out with a 50 mM potassium phosphate buffer pH 7.0, (experiment I) one peak of 3GT activity corresponding to a M_r of 78000 appeared, and a very broad shoulder of about M_r 20–40000. The results could be explained by aggregation or by assuming that the 3GT *in vivo* occurs as a oligomeric protein containing monomer units with M_r 26000. The findings are compatible with the earlier suggestion, based on kinetic studies, that the enzyme appears as a dimer [6]. Moreover, the inhibitory effect of ionic strength on 3GT activity (Table III) might be explained by a dissociation of an oligomeric structure to a less active monomer configuration. Anthocyanidin 3GT of *Silene* seemed also to exist as a dimer: M_r of 60000 and 125000 were reported [5]. The purified maize flavonol 3GT had a molecular weight of about 50000 [21].

Omitting β-mercaptoethanol in the buffers during extraction and elution did not affect the apparent molecular weights, which is an indication that the possible association of monomer units does not involve disulfide linkages.

Isoelectric point

The apparent iso-electric points of the two 3GT activities were determined by chromatofocusing of enzyme preparation. Again, we found no difference between the quercetin 3GT and the delphinidin 3GT. Both activities gave an identical elution profile, with a peak in a fraction corresponding to an iso-electric point of 5.2, which was earlier shown for the anthocyanidin 3GT [17]. The enzymes of an *an1an1* mutant (W78) and an *an2an2* mutant (W22), respectively, also showed maximum activity in the fractions with pH 5.2.

c. Identical antigenic structure of flavonol 3GT and anthocyanidin 3GT

Purification of 3GT

In order to study the structural relationship between flavonol 3GT and anthocyanidin 3GT by way of immunoprecipitation, partial purification of 3GT was carried out and this preparation was used to immunize a rabbit. In Table IV a summary of the purification procedure is given. Like the 3GT in maize, [22], the 3GT in *Petunia* was very unstable at

Table IV. Partial purification of 3GT.

Fraction	Protein mg	Activity nkat	Specific activity pkat/mg protein	Purification
PVPP, dialyzed	66	36.0	545	1
DEAE-cellulose	28	6.7	239	0.4
PBE 94	1	6.3	6300	11.6

PVPP = polyvinylpolypyrrolidon
The values of enzyme activities refer to standard assays with quercetin as substrate.

increasing degree of purity. Because of this loss of activity and in view of the reported low antigenicity of 3GT in maize [22], we did not attempt to purify the enzyme to homogeneity. SDS-electrophoresis of the partially purified preparation showed four main bands, the most intense band with M_r about 50000. The procedure resulted in a 12-fold increase in specific activity and the recovery of activity was about 20%. During purification, the ratio between quercetin 3GT activity and delphinidin 3GT activity did not change (results not shown).

Immunoprecipitation of enzyme

The anti-3GT serum was immobilized to protein A-Sepharose CL-4B and increasing amounts were tested for the capacity to precipitate flavonol 3GT and anthocyanidin 3GT in an enzyme preparation from a plant which synthesizes both anthocyanins and flavonols (V13). In Figures 4 and 5 the resulting titration curves are shown. Three flavonol-glucosylating activities are present in *Petunia* (Figure 1): quercetin 3GT, kaempferol 3GT and myricetin 3GT. These three flavonol glucosyltransferases precipitated with identical titration curves (Figure 4), which were also identical to those of the two anthocyanidin-glucosylating activities: delphinidin 3GT and cyanidin 3GT (Figure 5). The preparation used for immunization of the rabbit contained more than one protein. Therefore we cannot exclude the possibility that antiserum was raised against several hypothetical 3GT-enzymes. However, it is very unlikely that upon immunization of the rabbit these enzymes would give rise to antibodies which give identical titration curves. If flavonol 3GT and anthocyanidin 3GT were different enzymes, we would therefore expect the immunoprecipitation curves of the different 3GT activities to have different slopes. Since the curves were identical, the results give a very strong indication that there is only one 3GT, glucosylating all five substrates *in vivo*.

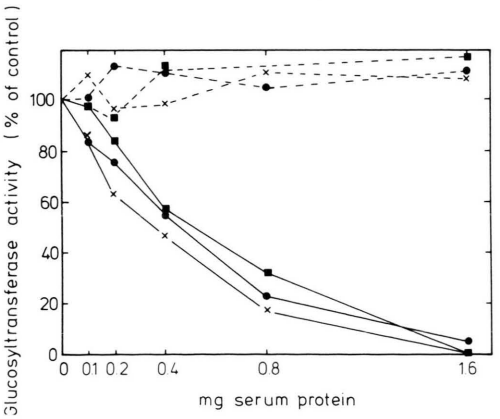

Fig. 4. Effect of pre-incubation with anti-3GT serum (——) and normal rabbit serum (– – –) on flavonol 3GT activities of the line V13. The control sample contained totally (in 100 µl) 143 pkat quercetin 3GT, 180 pkat kaempferol 3GT and 170 pkat myricetin 3GT. Enzyme assays contained 150 mM glycine-NaOH pH 9.2, 30 µM flavonol, 10% EGME (v/v). (●—●) = quercetin 3GT, (×—×) = kaempferol 3GT, (■—■) = myricetin 3GT.

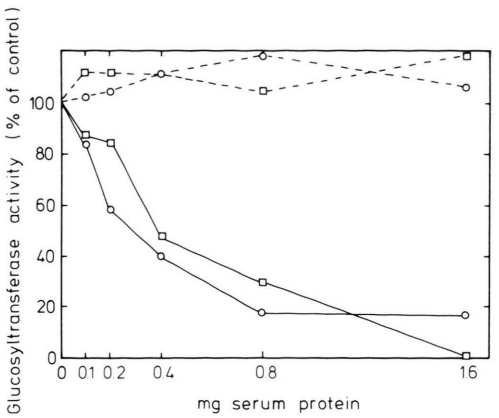

Fig. 5. Effect of pre-incubation with anti-3GT serum (——) and normal rabbit serum (– – –) on anthocyanidin 3GT activities of the line V13. The control sample contained 14 pkat delphinidin 3GT and 7 pkat cyanidin 3GT in 100 µl. Assays contained 15 mM potassium phosphate pH 7.4, 30 µM anthocyanidin, 10% EGME (v/v). (□—□) = delphinidin 3GT, (○—○) = cyanidin 3GT.

In order to investigate whether mutants with the genotype *an1an1* or *an2an2* contain a structurally different 3GT, the precipitation of 3GT activity by the anti-3GT serum was tested in extracts from one *an2an2* mutant (W22) and one *an1an1* mutant (W42). The results are illustrated in Figures 6 and 7, respectively. Precipitation curves of quercetin 3GT and delphinidin 3GT were identical in extracts from both mutants. Moreover, both mutants gave a curve similar to that of the line V13. The amounts of quercetin 3GT precipitated by 16 µl antiserum (1.6 mg protein) were alike: 135 pkat (V13), 123 pkat (W42) and 115 pkat (W22).

The lower level of 3GT activity observed in *an1an1* and *an2an2* mutants might be caused by synthesis of a structurally different, less active 3GT-enzyme. One would then expect more antiserum to

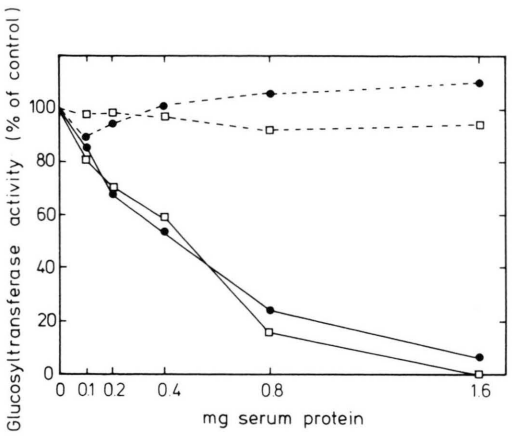

Fig. 6. Effect of pre-incubation with anti-3GT serum (——) and normal rabbit serum (– – –) on 3GT activities of the mutant W22 (*an2an2*). The control sample contained totally (in 100 µl) 123 pkat quercetin 3GT and 25 pkat delphinidin 3GT. Enzyme assays were carried out as described in the legends to Figs. 4 and 5. (●—●) = quercetin 3GT, (□—□) = delphinidin 3GT.

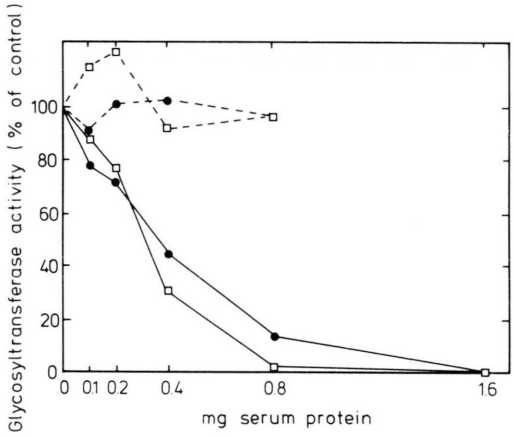

Fig. 7. Effect of pre-incubation with anti-3GT serum (——) and normal rabbit serum (– – –) on 3GT activities of the mutant W42 (*an1an1*). The control sample contained totally (in 100 µl) 123 pkat quercetin 3GT and 12 pkat delphinidin 3GT. Enzyme assays were carried out as described in the legends to Figs. 4 and 5. (●—●) = quercetin 3GT, (□—□) = delphinidin 3GT.

be needed to precipitate the same amounts of 3GT activity in the mutants as in the wildtype plants. Since amounts of 3GT activity precipitated by antiserum were equal, we conclude that 3GT of the mutants has the same specific activity as that of the wildtype and that there is no cross-reacting inactive 3GT present in the these mutants. Apparently, *an1an1* and *an2an2* mutants produce 3GT-enzyme that is structurally similar to that of wildtype plants, but in smaller amounts.

Why then do these mutants accumulate normal levels of flavonol 3-glucosides, but no or hardly any anthocyanin? Do they contain a structurally identical, yet functionally different 3GT? This seems implausible. Kho and co-workers [6] found no significant differences in kinetic parameters of the 3GT in mutants which were homozygous recessive for one of the genes *An1* or *An2*, as compared to the 3GT in wildtype flowers. These studies were performed with cyanidin as substrate; we extended them by using also a flavonol (quercetin) as substrate. The enzyme preparations obtained from the lines V13 (wildtype), W42 (*an1an1*) and W22 (*an2an2*) were used to study the 3GT activity as a function of the concentrations of delphinidin and quercetin, respectively. Incubations were carried out at identical conditions, at a pH of 7.4, with variations only in the protein concentration. The study of Kho [6] showed that 3GT did not exhibit Michaelis-Menten kinetics with regard to the anthocyanidin substrate. We are therefore not discussing Km-values. The glucosylation was maximal at substrate concentrations of 10 to 15 µM (quercetin and delphinidin), and half maximal values were obtained at 2 to 5 µM. No significant differences were found between the glucosyltransferases of the three preparations.

The incubations were carried out at a pH of 7.4, which is a suboptimal pH-value for the glucosylation of quercetin (Fig. 2); yet in all cases the glucosylation of flavonol was 4–6 times as fast as the glucosylation of delphinidin. These observations provide an explanation for how, in spite of the low 3GT-activities, the mutant lines accumulate wildtype levels of flavonol 3-glucosides. The residual activity in these mutants might be sufficient for production of flavonol 3-glucosides, but limiting for a normal biosynthesis of anthocyanidin 3-glucosides. Moreover, a higher affinity of the 3GT for the flavonol substrate *in vivo* cannot be excluded.

In conclusion, all evidence supports the notion that flavonol 3GT and anthocyanidin 3GT in *Petunia hybrida* is one and the same enzyme, exhibiting a higher activity towards flavonols than to anthocyanidins. However, the literature mentioned in the introduction suggests that this situation is not

universal. Apparently, flavonol-specific 3GTs and anthocyanidin-specific 3GTs exist as well as 3GTs which can glucosylate both flavonols and anthocyanidins. Between these three classes of 3GTs we could expect significant structural differences. This idea was confirmed by the fact that the anti-3GT serum from *Petunia* did not precipitate the anthocyanidin-specific 3GT from *Silene* (van Brederode, unpublished results).

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