UDP-Glucose: Anthocyanidin/Flavonol 3-0-Glucosyltransferase in Enzyme Preparation from Flower Extracts of Genetically Defined Lines of *Matthiola incana* R. Br.

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In flower extracts of *Matthiola incana* an enzyme catalyzing the transfer of glucose from UDP-glucose to the hydroxyl group at 3-position of anthocyanidins and flavonols was demonstrated. The pH-optimum of this reaction is at pH 8.5 for pelargonidin and pH 9.5 for quercetin as substrate. The reaction is inhibited by both substrates above 10 nmol per assay. The enzyme is highly active, within 30 sec 3 nmol of 3-glucosides were formed. At 30 °C the enzyme is stable for hours and at −20 °C months. Besides UDP-glucose, TDP-glucose is a suitable glucosyl-donor, but with a reduced (70%) reaction rate. Enzyme activity is clearly inhibited by Fe^{2+} and Cu^{2+} ions, and by diethylpyrocarbonate. Acyanic or pale coloured mutants of several genes interfering with anthocyanin synthesis after dihydroflavonol formation show a more or less drastically reduced enzyme activity (5–40%). But none of these genes can be regarded as the structural gene for the 3-glucosyltransferase. The influence of these genes on enzyme activity and flower colour is discussed.

Introduction

Anthocyanins play an important role as pigments in flowers and other plant parts. Generally they occur as glycosides. One of the simplest types are the 3-glucosides, which are substrates for further glycosylations and other modifications at different positions. Enzymatic formation of anthocyanidin 3-0-glucosides was first demonstrated in pollen of *Zea mays* [1]. Enzyme activity was also found in flowers of *Silene dioica* [2], *Petunia hybrida* [3, 4] and *Matthiola incana* [5], in cell cultures of *Haplopappus gracilis* [6], in seedlings of *Brassica oleracea* [7] and in *Tulipa cultivars* [8]. In all cases uridine diphosphate-D-glucose serves as glucosyl-donor. With the exception of the transferase found in *Silene dioica* the known transferases are able to use anthocyanidins as well as flavonols as substrate for the glucosylation reaction. The enzyme is therefore designated as UDP-glucose 3-0-flavonoid glucosyltransferase (3GT).

A clear genetic control of the 3GT activity has been demonstrated in *Zea mays* [9, 10]. Mutants with recessive alleles of the gene *bz* lack 3GT activity. Moreover, the gene dosage relationship found for different numbers of functional alleles suggest that *bz* is the structural gene for 3GT [10]. In *Silene dioica* and *Petunia hybrida* some genes which interfere with the anthocyanin pathway between dihydroflavonol and anthocyanidin also influence 3GT activity. In the respective mutants a 3GT activity of only 5–20% of the wildtype was found to be present [2, 3].

In context of our efforts to elucidate the last steps in the anthocyanin pathway the 3GT reaction is of special interest. On the one hand the anthocyanidins formed by as yet still unknown reactions from leucoanthocyanidins are only stable after glycosylation in 3-position. On the other hand the anthocyanidin 3-glucosides are the substrates for further modifications of the anthocyanin molecule by glycosylations and acylation.

The present work reports on the characterization of the 3GT from flowers of *Matthiola incana* and on the influence on enzyme activity of several genes interfering with the late steps of anthocyanin biosynthesis.

Material and Methods

Plant material

The investigations included several genetically defined lines of *Matthiola incana* R. Br. The genotypes,

Abbreviations: 3GT, UDP-glucose: anthocyanidin/flavonol 3-0-glucosyltransferase; UDPG, uridine-diphosphate-glucose; Kpi, potassium phosphate; PVP, polyvinylpyrrolidone; EGME, ethyleneglycolmonoethylether; EDTA, ethylenediaminetetraacetic acid.

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major flavonoid content and flower colour are compiled in Table I. The plants were cultivated in the greenhouse or in the experimental garden of our institute.

Chemicals and reference compounds

Pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), paeonidin (Pn), kaempferol (Km) and quercetin (Qu) are obtained from Roth (Karlsruhe, FRG). UDP-glucose and other activated sugars are purchased from Sigma (Taufkirchen, FRG). UDP[U^{14}C]-glucose was obtained from Amersham-Buchler (Braunschweig). The 3-glucosides of anthocyanidins and flavonols came from our laboratory collection. Cyanidin- and pelargonidinchloride were polluted to a high degree. Therefore, they were purified by paperchromatography (Schleicher & Schüll 2043b) using the solvent system BAW (*n*-butanol : acetic acid : H_2O = 6:1:2).

Enzyme preparation

All steps were performed at 4 °C. 1.0 g petals at stage III or IV of flower development [16] were homogenized in a prechilled mortar together with 1.0 g quartz sand, 0.5 g PVP (Polyclar At, Serva) and 6 ml 0.1 M Kpi buffer, pH = 7.0, containing 5 mM 2-mercaptoethanol. The homogenate was transferred to Micro test tubes (Eppendorf) and centrifugated for 10 min at about 10000 × *g*. The supernatants were pooled and again centrifugated as described before. The crude extract was passed throught a Sephadex G-50 column (bed volume = 1 ml) to free it from phenolic compounds and other low molecular weight substances.

Enzyme assay

The reaction mixture contained in a total volume of 100 µl: 80 µl 0.05 M Kpi buffer, pH = 7.0, 0.5 mM 2-mercaptoethanol, 5 µl UDPG (70 nmol), 10–20 µg protein and 5 µl anthocyanidin resp. flavonoid (30 nmol) solved in EGME. The reaction was started by the addition of anthocyanidin. After incubation for 3 min at 35 °C, the reaction was terminated by adding 100 µl chloroform : MeOH = 2:1 (0.5% HCl), resulting in a Folch partition [17]. For the radioactive tests [^{14}C]UDPG was used (70 nmol, 1850 Bq).

Analytical methods

Two methods for analysing the reaction mixture were used. First HPLC and second TLC. In the Folch partition the anthocyanins were concentrated in the upper phase, 50 µl of this phase were injected into a high performance liquid chromatograph (Spectra Physics SP 8700) equipped with a Spherisorb ODS II (3 µm RP 18) column (Bischoff, Leonberg) with the dimensions 125 × 4.6 mm and a precolumn of 10 × 4.6 mm (5 µm RP 18). Substances were detected by a variable wavelength detector (Knauer, FRG) at 530 nm resp. 350 nm. These wavelengths were chosen as a compromise. At 530 nm the anthocyanidins (cyanidin, delphinidin, paeonidin and pelargonidin) and their 3-glucosides absorb well and so they could be detected together in the same reaction mixture. The same is valid for the flavonols at 350 nm. Concentrations were determined by measuring the peak area (Spectra Physics, Integrator SP 4150). The absolute value was gained by comparing the peak area of each substance to its standard curve. The elution system for anthocyanins consisted of two solvents, A = 1.5% H_3PO_4 in CH_3CN and B = 0.5% H_3PO_4 in water. The linear gradient with the procentual portion of A in A + B was varied in 6 min from 10% to 100%, and then kept for 1.5 min at 100%. The separation was performed at room temperature with a flow rate of 1.5 ml × min^{-1}. The elution system for flavonols, their 3-glucosides and other flavonoids consisted of solvent A and B, A = MeOH and B = 5% HCOOH in H_2O. Here the linear gradient started with 15% A in A + B and varied in 10 min to 70%, then from 70%–100% A in 2 min, where it rested for 1 min. The separation was performed at room temperature with a flow rate of 0.7 ml × min^{-1}. The retention times of the aglycones and their 3-glucosides are listed in Table II. For TLC analysis the concentrate was charged on cellulose plates (Schleicher & Schüll) together with the appropriate references using the following solvent systems HOAc–HCl (H_2O : HOAc : HCl = 82:15:3) and BAW. The R_f-values are summarized in Table II. This solvent system was also used for separation of the upper phase of the Folch partition in tests with labelled UDPG.

The plates were scanned for radioactivity and the 3-glucosides scraped off and counted in Unisolve (Zinsser Analytic, Frankfurt) in a scintillation counter (1219 g Rackbeta, LKB). Standard procedures were used for identification of the flavonoid-3-glucosides by acid hydrolysis and spectral analysis [19].

The protein content of the enzyme extracts was estimated by the method of Bradford [19].

Determination of the pH-optimum

The enzyme assays were carried out in a mixture of 100 µl volume in total: 85 µl 0.1 M Britton Robinson buffer between pH 6.0–10.0, 0.05 mM 2-mercaptoethanol, 5 µl protein (5 µg protein), 5 µl UDPG (70 nmol) and 5 µl flavonoid (30 nmol).

Determination of substrate saturation

The total volume of the reaction mixture was 100 µl with a flavonoid content of 3.5–90 nmol, 85 µl 0.05 M Kpi buffer, 10 µl protein (10 µg protein) and 5 µl UDPG (70 nmol).

Results

Wild-type lines of *Matthiola incana* produce a complex pattern of acylated pelargonidin or cyanidin 3-sambubioside-5-glucosides in the flowers. To characterize enzymatic glucosylation in the 3-position separately from other glycosylation reactions the mutant lines 08 and 16 were used for the investigations. These lines produce mainly 3-glucosides of cyanidin (line 08) or pelargonidin (line 16) in the flowers (Table I).

Gene b controls the activity of flavonoid 3′-hydroxylase [11]. Flowers with dominant alleles produce cyanidin and those with recessive alleles pelargonidin. The activity of chalcone synthase is controlled by gene *f* [12]. Gene *e* concerns the enzymatic reduction of dihydroflavonols to flavan 3,4-diols [13, 14]. Gene *g* and *z* also interfere with anthocyanin synthesis after dihydroflavonol formation but their real action is still unknown. Recessive alleles of gene *e*, *f* or *g* prevent anthocyanin synthesis, while the multiple allele *g′* of the gene *g* and recessive alleles of the gene *z* allow anthocyanin synthesis but with a reduced rate [15].

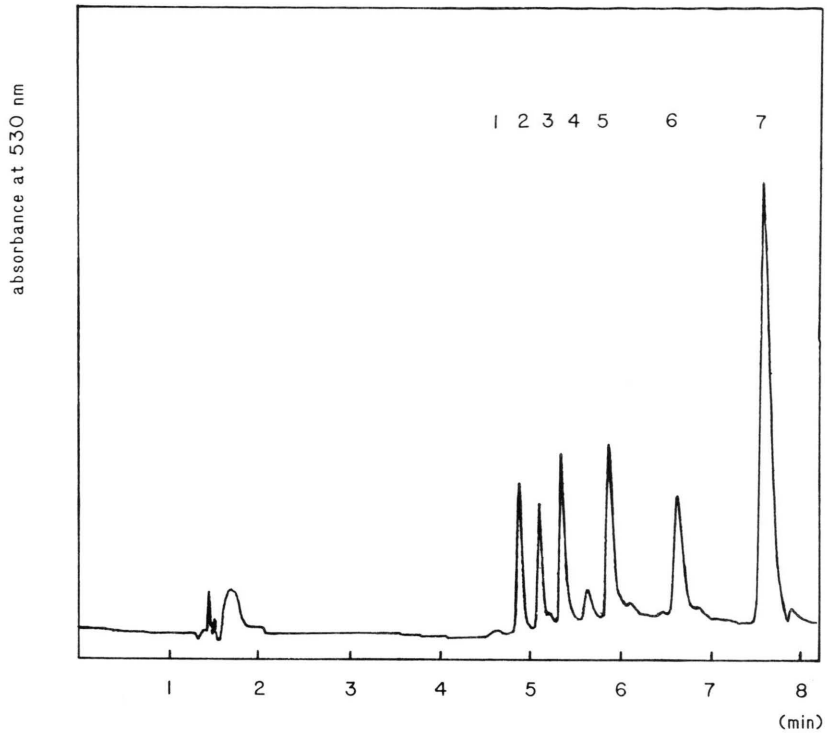
When enzyme extracts of these lines were incubated with UDPG and pelargonidin or cyanidin, one product was detected after TLC or HPLC of the reaction mixture. Depending on the anthocyanidin used as substrate the product comigrated with pelargonidin- or cyanidin-3-glucoside, respectively. The formation of the respective anthocyanidin 3-glucosides was further confirmed by spectrophotometric analysis and controlled acid hydrolysis.

No difference in substrate specificity and reaction rate of the enzyme extracts from cyanidin or pelargonidin containing flowers was observed (Table I). Even delphinidin and paeonidin which are naturally not present in flowers of *Matthiola incana* were found to be glucosylated in 3-position. Moreover, when pelargonidin, cyanidin and delphinidin were present as substrates in the same reaction mixture, they were glucosylated in 3-position without any preference to one of them (Fig. 1). Besides anthocyanidins, flavones, flavanones, dihydroflavonols and flavonols were tested as substrates for glucosylation. Flavonols were found to be glucosylated in 3-position even at a higher rate than anthocyanidins. Even, when pelargonidin and quercetin were present as substrates in the same reaction mixture, both were

Table I. Genotypes, main flavonoid contents, flower colour and 3GT activity of genetically defined lines of *Matthiola incana*.

Line	Genotyp *bb/ee/ff/zz/g g*	Predominant flavonoid	Colour of petals	3GT Activity Pelargonidin [nmol 3-glu.* /µg protein]	3GT Activity Quercetin [nmol 3-glu.* /µg protein]
08	++/++/++/++/+ +	cyanidin 3-glycosides	brown violet	0.9 =100%	2.4 =100%
16	*bb*/++/++/++/+ +	pelargonidin 3-glycodides	brown violet	0.9 =100%	2.4 =100%
17b	*bb/ee*/++/++/+ +	kaempferol glycosides	white	0.24= 27%	0.96= 40%
18b	*bb*/++/*ff*/++/+ +	hydroxycinnamic acid glu.*	white	0.76= 84%	2.2 = 92%
19b$^+$	++/++/++/++/*g g*	kaempferol glycosides	white	0.06= 7%	0.24= 10%
20	++/*ee*/*ff*/++/+ +	not tested	white	0.32= 36%	0.94= 39%
21	++/*ee*/++/++/*g g*	kaempferol glycosides	white	0.09= 10%	0.36= 15%
22	++/++/*ff*/++/*g g*	not tested	white	0.05= 5%	0.14= 6%
24	++/++/++/++/*g′ g′*	cyanidin glycosides	pale violet	0.17= 19%	0.5 = 21%
25	++/++/++/*zz*/+ +	cyanidin glycosides	pale violet	0.32= 35%	0.84= 35%
28	++/++/++/*zz*/*g′ g′*	not tested	white	0.07= 8%	0.31= 13%

* glu. = glucoside.



1: Dp 3-G, 2: Cy 3-G, 3: Pg 3-G, 4: impurity, 5: Dp, 6: Cy, 7: Pg

Fig. 1. HPLC analysis of the 3-GT reaction mixture with Pg, Cy, and Dp as substrates.

Table II. R_f-value and retention times of substrates and products.

Flavonoid	R_f-value in BAW	× 100 HOAc-HCl	R_t-value [min]
PG 3-G*	40	36	5.3[a]
Cy 3-G	25	25	5.0[a]
Dp 3-G	16	16	4.8[a]
Pn 3-G	–	–	5.3[a]
Pg	–	20	7.5[a]
Cy	–	11	6.6[a]
Dp	–	5	5.9[a]
Pn	–	–	7.7[a]
Km 3-G	71	40	12.3[b]
Qu 3-G	60	32	11.0[b]
Km	85	–	15.0[b]
Qu	72	–	13.6[b]

3-G* = 3-glucoside.
[a] In elution system A = 1.5% H_3PO_4 in CH_3CN
B = 0.5% H_3PO_4 in H_2O.
[b] In elution system A = MeOH
B = HCOOH.

converted into the respective 3-glucosides. The reaction rate of pelargonidin and quercetin was identical with that observed in reactions where only one of them served as substrate. The 3-glucosides formed, were identified as described above. The other flavonoid classes were neither glucosylated in 3-position nor in 7-position, although 7-O-glucosides of flavanones and dihydroflavonols are known to be present in flowers of *Matthiola incana* [20, 21]. In order to get a notion of the stability of the anthocyanidins and flavonols under assay conditions their disintegration was followed at their absorption maxima by measuring the peak area of the HPLC inject. Flavonols such as quercetin reveal no considerable decrease of the peak area, whereas the anthocyanidins are destroyed within minutes at different rates with pelargonidin being the most stable one (Fig. 2). Therefore, pelargonidin and quercetin were used for the further characterization of the reaction.

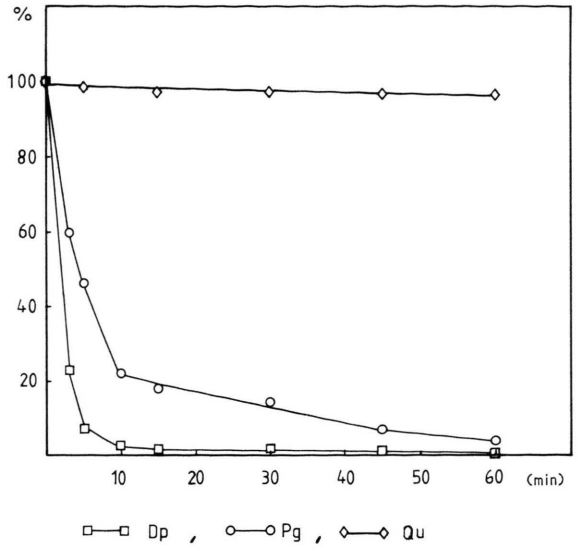


Fig. 2. Time-course of disintegration of pelargonidin chloride delphinidin chloride and quercetin in 0.05 m Kpi (pH = 7.0).

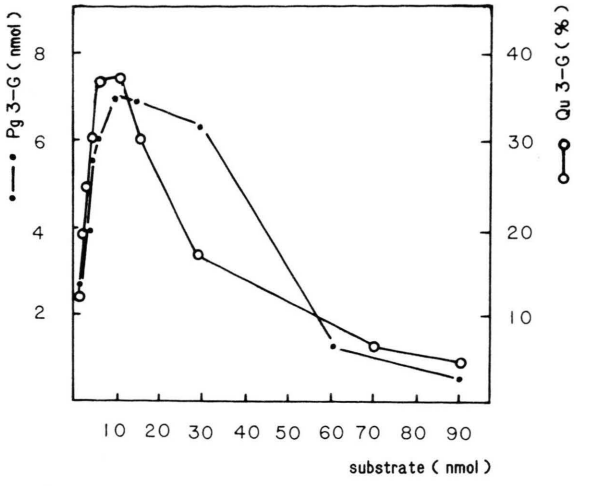


Fig. 3. Effect of substrate concentration on the glucosylation reaction.

The crude extracts prepared with PVP still contained visible amounts of anthocyanins. Purification by passing the extracts through a Sephadex G-50 (fine) column removed these pigments. No considerable change in enzyme activity could be observed comparing the crude extracts with the purified enzyme preparation but HPLC analysis was easier without these contaminating anthocyanins. The protein content of the G-50 eluates generally amounted to 1 mg × ml^{-1}.

The reaction rate increased proportional with protein concentration up to 30 µg protein per assay. Linearity with time could be obtained up to 5 min with pelargonidin and 10 min with quercetin as substrate. A sharp optimum for concentration of both substrates was determined. Strong inhibition of the reaction rate was found when the optimal concentration of 10 nmol substrate was exceeded (Fig. 3). In contrast, increasing the concentration of UDPG over 70 nmol did neither decrease, nor increase the reaction rate.

In Britton-Robinson buffer the enzyme exhibited a pH-optimum at pH 8.5 for pelargonidin and at 9.5 for quercetin as substrate. The difference between the pH-optima is due to the fact that pelargonidin is quicker disintegrated under basic conditions than under neutral conditions while quercetin is stable. For that reason the investigations were also not performed at the optimal pH but at pH 7.0 where the reaction rate was about 60% pelargonidin and 65% for quercetin in comparison to the pH-optimum.

With quercetin as substrate enzyme activity was compared in different buffers with the result that highest conversion to the 3-glucoside was gained in 0.1 M Kpi. The conversion rate in 0.1 M NaOH/Glycin and Britton-Robinson was about 50% compared to Kpi, while 0.1 M Tris/HCl gave 75%.

For a clear quantification of the reaction rate with pelargonidin and quercetin als substrate, the tests were performed with [^{14}C]UDPG and the reaction mixture separated by TLC. Scintillation counting of the 3-glucoside bands formed from pelargonidin or quercetin, respectively, gave 20.3 Bq and 56.6 Bq with 10 µg protein per assay.

Besides UDPG other activated glucose compounds as ADPG, TDPG, GDPG and CDPG were tested as donors of the sugar moiety. Only TDPG was found to serve as a glucose donor, but the 3-glucosides were formed at a lower rate (75%) compared to UDPG. Moreover, only the transfer of glucose could be observed. Other activated sugars as UDP-xylose, UDP-mannose and UDP-galactose failed as donors.

The enzyme is highly active, within 0.5 min about 10% of the substrate are converted into 3-glucoside at 35 °C. A temperature optimum was found at about 37 °C, when the reaction time amounted to

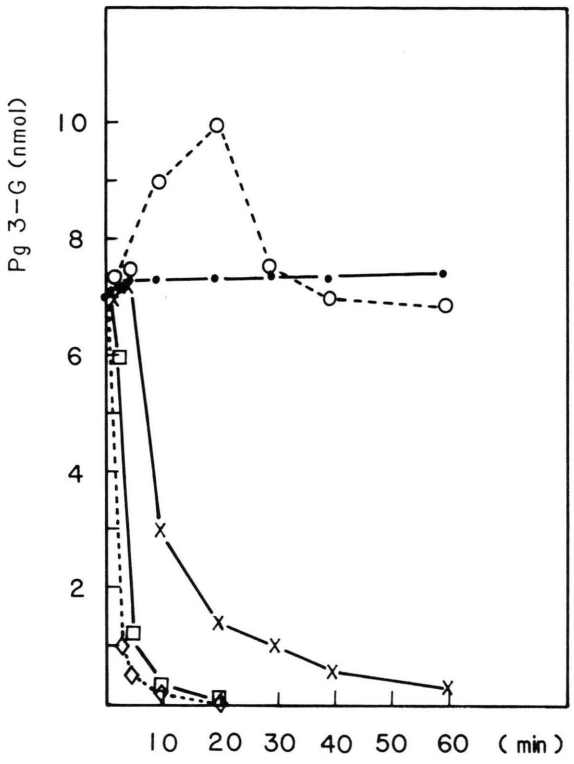


Fig. 4. Enzyme stability as a function of time at different temperatures. Product formation is a value for enzyme stability. ●——● 30 °C, ○---○ 40 °C, x——x 50 °C, □——□ 60 °C, ◇---◇ 70 °C.

3 min. But the enzyme was active even at 0 °C with a rate of 20% compared to 37 °C. Preincubation of extracts at different temperatures revealed stability of the enzyme at 30 °C and 40 °C for at least 60 min. At 40 °C an initial activation was observed which decreases to the original level of enzyme activity after 30 min. At higher temperatures the enzyme is very rapidly destroyed (Fig. 4). The measurements of enzyme stability at different temperatures are valid for pelargonidin and quercetin. Crude extracts and G-50 eluates could be stored at −20 °C for several months without loss of 3 GT activity. Addition of glycerol and BSA was not necessary. Several ions and inhibitors of enzyme reactions were tested. Ions as Mg^{2+}, Co^{2+} and Ca^{2+} stimulate the reaction slightly, whereas Fe^{2+}, Cu^{2+} and Zn^{2+} show a clear inhibitory effect, Cu^{2+} is the strongest inhibitor (Table III). Substantial inhibition was also observed by addition of diethylpyrocarbonate and *para*-chloromercuribenzoate (Table III). The effects of the investigated substances were nearly the same for pelargonidin and quercetin as substrate (Table III).

Table III. Effect of metal ions and inhibitors on 3-glucosylation of pelargonidin and quercetin.

Addition	3-Glucosyltransferase activity [%]	
	Pelargonidin	Quercetin
None	100	100
1 mM KCl	100	100
1 mM K_2SO_4	100	100
1 mM KCN	100	100
1 mM $CaCl_2$	105	110
1 mM $MgCl_2$	110	110
1 mM $CoCl_2$	111	110
1 mM $FeCl_2$	37	40
1 mM $FeSO_4$	48	50
1 mM $CuSO_4$	23	15
1 mM $ZnSO_4$	87	73
1 mM $ZnCl_2$	79	80
2 mM EDTA	93	95
2 mM DDC	115	105
1 mM DPC	60	45
0.2 mM *p*-CMB	70	–

EDTA : ethylenediaminetetraacetic acid;
DDC : diethyldithiocarbamate;
DPC : diethylpyrocarbonate;
p-CMB : *p*-chloromercuribenzoate.

The influence of the genes *e, f, g* and *z* on enzyme activity was studied. In the presence of recessive alleles of the gene *f* (line 18) only a low difference of the enzyme activity of the wild-type was observed, whereas in enzyme preparations from lines homozygous recessive for *e* (line 17) or *z* (line 25) a substantial reduction was found (Table I). A similar clear decrease in enzyme activity was observed in flower extracts of line 24 with the multiple allele *g'* of the gene *g*, while in preparations from flowers of line 19 with recessive alleles of this gene enzyme activity was even reduced to 7–10% compared to the wild-type (Table I). The double mutants *eeff* (line 20), *eegg* (line 21) and *ffgg* (line 22) showed no additive effect, whereas in flower extracts of the double mutant *zzg'g'* (line 28) enzyme activity was clearly lower than in the respective single mutants (Table I).

Discussion

Anthocyanidins are most probably not naturally present in flowers and other plant parts, because of their general instability and great insolubility. They should therefore not be regarded as endproducts like flavones or flavonols but rather as intermediates. The first stable and naturally present products are the anthocyanidin 3-glycosides with the 3-glucosides as the most common compounds. Thus, the 3GT is not a modifying enzyme but catalyzes a very important step in the biosynthetic pathway of anthocyanins. In this context the high enzyme activity found in flower extracts of *Matthiola* lines with strong anthocyanin content is not unexpected, because it makes sure that every anthocyanidin molecule formed from leucoanthocyanidins is immediately transformed to the 3-glucoside and so stabilized.

The high enzyme activity was also of great advantage for the measurements performed. Because visible amounts of 3-glucosides were formed within a very short incubation time, the reaction rate was only slightly influenced by the decomposition of the anthocyanidins under assay conditions. Moreover, quantification could easily be achieved by HPLC analysis of the reaction mixture. Thus, the use of radioactively labelled UDPG was only necessary for a better comparison between the reaction rates of anthocyanidins and flavonols.

The determination of the pH-dependence for 3GT revealed a clear maximum at pH 8.5 for pelargonidin and pH 9.5 for quercetin. Similar high pH-optima have been reported for the 3GT of other plants [3, 6, 7]. The broad pH-optimum found at former investigations on 3GT in *Matthiola incana* was a result of determination under unsuitable assay conditions. The strong substrate inhibition with both pelargonidin and quercetin is not due to pollution of these compound. Comparison of the commercial samples with purified (HPLC) preparations showed no considerable difference in reaction rate. Strong substrate inhibition was also reported for the 3GT from red cabbage seedlings and from *Haplopappus* [6, 7]. In agreement with the 3GT of many other plants, the enzyme of *Matthiola incana* has a distinct position specificity and uses anthocyanidins and flavonols as substrates. The latter result raised the question of whether one and the same enzyme is responsible for 3-glucosylation of anthocyanidins and flavonols. Detailed studies of this question with enzyme preparations of *Petunia hybrida* support the idea of a common identity of anthocyanidin and flavonol 3GT [25]. In *Matthiola incana,* the genes affecting 3GT activity show comparable reductions of the reaction rate for pelargonidin and quercetin as substrate. Moreover, the ions and inhibitors tested exhibit similar effects on the conversion of both substrates to the respective 3-glucosides. Furthermore, the thermal inactivation curves of the enzyme activity are also comparable for pelargonidin and quercetin. These results suggest that in *Matthiola incana* 3-glucosylation of anthocyanidins and flavonols is also catalyzed by the same enzyme. The difference found in the pH-optima for pelargonidin and quercetin cannot be regarded as an evidence for two different 3GT enzymes. They are caused by the different chemical properties of anthocyanidins and flavonols under basic conditions. It is notable that dihydroflavonols were not glucosylated, although they possess a hydroxyl group in 3-position. Moreover, preliminary studies on leucoanthocyanidins showed that these compounds also did not serve as substrates for 3-glucosylation (Heller and Forkmann, unpublished results). This supports the conclusion that glucosylation is the last step in anthocyanidin biosynthesis [22, 23].

In *Silene dioica* and in *Petunia hybrida,* recessive mutants of some genes localized in the biosynthetic pathway between dihydroflavonol and anthocyanidin have a clearly reduced 3GT activity [2–4]. A similar reduction of 3GT activity was now found for mutants of the genes *e, g* and *z* which also interfere with the

anthocyanin pathway after dihydroflavonol formation. None of these genes can be regarded as the structural gene for 3GT. The gene *e* was recently correlated with the activity of dihydroflavonol 4-reductase [14]. The activity of this enzyme is surprisingly also controlled by the gene *g* but genetic studies, supplementation experiments and the data for 3GT suggest that this gene exerts most probably a regulatory function on gene *e* and obviously on the structural gene coding for 3GT [13, 14]. Thus, the recessive mutant (*gg*, line 19) lacks 4-reductase activity and possesses only about 10% 3GT activity, while the mutant with the multiple allele *g'* (line 24) forms some anthocyanins in the flowers and, in agreement with this fact, shows a moderate activity of 4-reductase (Forkmann, unpublished) and 3GT.

A similar relation between pale flower colour and reduced 3GT activity is found for the gene *z*. Moreover, the double mutant (*g'g'zz*, line 28) shows not only a clearly lower 3GT activity than the single mutants, but also produces completely white flowers.

Nevertheless, in none of the white flowering mutant lines the more or less strong reduction of 3GT activity is directly responsible for the lack of anthocyanins. The remaining activity of about 5–10% should still be sufficient for the formation of visible amounts of anthocyanins in the flowers. It also can be excluded that the existence of flavonols which are obviously better substrates for the 3GT reaction disturb anthocyanin synthesis, because wild-type lines contain anthocyanins as well as flavonols. Moreover, flavonol synthesis is already terminated when the bulk of anthocyanin synthesis occurs [24].

A further notable fact is that the mutant blocked in chalcone synthase activity (line 18, recessive *f*) shows nearly wild-type activity of 3GT. This indicates that no anthocyanidins or other intermediates of flavonoid biosynthesis are needed for 3GT induction. The influence of several genes on 3GT activity found in *Matthiola incana* and other plants seems as yet best rationalized by the assumption that the enzymes for the last steps in anthocyanin biosynthesis are connected together in a functional complex, so that a defect of one of them affects the other in their action. A verification of this idea can only be achieved by the elucidation of the still unknown last steps in anthocyanin biosynthesis, concerning the reaction sequence from leucoanthocyanidins to anthocyanidins. Such work is now in progress.

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