Identification, Properties and Genetic Control of UDP-L-Rhamnose: Anthocyanidin 3-*O*-Glucoside, 6″-*O*-Rhamnosyltransferase Isolated from Retals of the Red Campion (*Silene dioica*)*

John Kamsteeg[a], Jan van Brederode, and Gerrit van Nigtevecht
Department of Population and Evolutionary Biology, University of Utrecht, The Netherlands

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An enzyme catalyzing the transfer of the rhamnosyl moiety of UDP-L-rhamnose to the 6-hydroxyl group of the 3-*O*-bound glucose of anthocyanidin 3-*O*-glucosides has been demonstrated in petal extracts of *Silene dioica* plants. The enzyme activity is controlled by a single dominant gene *N*; no rhamnosyltransferase activity is found in petals of *n*/*n* plants. The 60-fold purified rhamnosyltransferase exhibits a pH optimum of 8.1, has a molecular weight of about 45000 daltons, is stimulated by the divalent metal ions Mg^{2+}, Mn^{2+} and Co^{2+}, and has a "true *Km*" value of 0.09 mM for UDP-L-rhamnose and 2.2 mM for cyanidin 3-*O*-glucoside. Pelargonidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside can also serve as acceptor. The enzyme can also catalyze the rhamnosylation of anthocyanidin 3,5-diglucosides although at reduced rate. The biosynthetic pathway for the synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside in petals of *S. dioica* is discussed.

Introduction

The glycosylation of anthocyanins in petals of the Red Campion (*Silene dioica*) is governed by the genes *M* and *N*. Dominant alleles of both genes are required for the synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside, the anthocyanin present in petals of population plants all over Europe. Gene *M* governs the glucosylation of the 5-position of anthocyanidin 3-rhamnosylglucosides, whereas gene *N* controls the attachment of rhamnose to the glucose at the 3-O-position. Previous biochemical and genetical studies [1–3] have demonstrated that gene *M* controls the formation of UDP-glucose: anthocyanidin 3-rhamnosylglucoside, 5-O-glucosyltransferase. Although no variant has been found of the gene which controls the 3-O-glucosylation of cyanidin, the enzyme which catalyzes this step (UDP-glucose: anthocyanidin 3-O-glucosyltransferase) has demonstrated to be present in petals of *S. dioica* plants [4]. In this paper we describe the identification and properties of the enzyme controlled by gene *N*.

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[a] Present address: Department of Human Ecology, P. O. Box 8060, Wageningen, The Netherlands.

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Results

Enzymatic synthesis of cyanidin 3-rhamnosylglucoside

Incubation of UDP-L-rhamnose, labelled uniformly in the rhamnose moiety, and cyanidin 3-O-glucoside with the supernatant of a crude petal homogenate of *Silene dioica* plants of the genotype *m*/*m* *N*/*N*, resulted in incorporation of radioactivity into cyanidin 3-rhamnosylglucoside. When trichloroacetic acid was added before incubation, or when the enzyme preparation was omitted in the assay, no incorporation was observed. Without the addition of cyanidin 3-O-glucoside to the supernatant of the crude homogenate, 20% of the synthesis occurred, indicating that the supernatant contained endogenous cyanidin 3-O-glucoside. After removal of this endogenous substrate by PVP-chromatography and gel-filtration, no incorporation of radioactivity into cyanidin 3-rhamnosylglucoside was found when cyanidin 3-O-glucoside was omitted.

Identification of reaction product

The enzymatically formed product could not be distinguished chromatographically from cyanidin 3-rhamnosylglucoside by the following criteria:

1. On Whatman III the product chromatographed with cyanidin 3-rhamnosylglucoside in the solvent systems BuHCl and HOAc.

2. After two-dimensional chromatography of a mixture of the enzymatically formed product and authentic cyanidin 3-rhamnosylglucoside, in the solvent systems BAW and HCl, respectively, the position of the radioactive compound on the chromatogram coincided with that of cyanidin 3-rhamnosylglucoside.
3. When a mixture was purified by chromatography on Whatman III in the solvent systems BAW, HCl and Forestal, used in that order, and on silica gel G thin layer in the solvent system EFW, the specific activity rose from 2380 to 4138 cpm/μmol [2].
4. Partial hydrolysis according to Lynn and Luh [6] of the radioactive product demonstrated that only the rhamnose moiety was labelled. The radioactivity was only found in the remaining cyanidin 3-rhamnosylglucoside spot but not in the cyanidin 3-glucoside and cyanidin spot. From the sugars liberated only rhamnose was labelled.

Conditions for enzyme activity and enzyme properties

In crude extracts, the presence of soluble PVP, a reducing agent and Triton X-100 were necessary for maximal activity. In the Sephadex G-50 protein fraction, however, PVP could be omitted. β-Mercaptoethanol or cysteine could be used as reducing agents. Tween 20, cetrimide or 2-methoxyethanol (EGME) could not act as substitute for Triton X-100 (Table I); in fact they inhibited the reaction rate.

The enzyme was further purified as outlined in Table II. The activity of the concentrated Sephadex G-50 protein fraction diminished by 25% after storage for 3 months at −20 °C. Repeated freezing and thawing of the protein preparation led to a rapid decline in activity. When stored at 4 °C the enzyme lost 80% of its activity in one week.

Protein and time linearity

The amount of cyanidin 3-rhamnosylglucoside formed from cyanidin 3-O-glucoside and UDP-L-

Table I. Influence of detergents, reducing agents and soluble PVP on UDP-L-rhamnose: anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase activity in crude extracts of petals of *S. dioica* plants.

Emulgator	Final concentration [%]	Reducing agent [20 mM]	PVP [5%]	Percent total activity
Triton X-100	0.01	β-mercaptoethanol	+	100
Triton X-100	0.01	cysteine	+	92
Triton X-100	0.01	none	+	48
Triton X-100	0.01	β-mercaptoethanol	–	68
–	–	β-mercaptoethanol	+	78
–	–	–	+	13
–	–	β-mercaptoethanol	–	26
Tween 20	0.02	β-mercaptoethanol	+	37
EGME	0.04	β-mercaptoethanol	+	58
Cetrimide	0.01	β-mercaptoethanol	+	57

One gram petals was homogenized in 1 ml of a 50 mM potassium-sodium phosphate buffer (pH 7.5) together with the additions indicated, and the preparation was centrifuged for 10 minutes at 38 000 × *g*. To 25 μl of the supernatant, 48 nmol UDP-L-[U-^{14}C]rhamnose (S.A. 3 Ci/mol), 73 nmol cyanidin 3-O-glucoside and 50 nmol $MnCl_2$ were added. The reaction mixture was incubated for 10 minutes at 30 °C.

Table II. Purification of UDP-L-rhamnose: anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase from petals of *S. dioica* plants.

Protein fraction	Volume [ml]	Protein [mg/ml]	Total activity [units[a]/min]	Specific activity [units/min/mg prot.]	Enzyme purification
Homogenized crude extract	15	9.3	27.9	0.20	1
Supernatant crude extract	12	6.8	22.9	0.28	1.4
Concentrated PVP-eluate	21.6	1	86.1	7.79	39.9
Concentrated G-50 eluate	39.2	0.25	115.8	11.82	59.1

[a] One unit is the amount of enzyme which catalyzes the formation of 1 nmol cyanidin 3-rhamnosylglucoside in one minute at 30 °C in the standard assay system.

rhamnose was found to be proportional to added enzyme and to time for periods up to 15 minutes at pH 7.5.

pH optimum

The enzyme exhibited a pH optimum of 8.1 with half-maximum velocities of pH 6.1 and 8.8. The enzyme had an identical pH curve both in glycylglycine buffer and in phosphate buffer. The skewness of the curve is probably due to protein denaturation and the instability of anthocyanins at higher pH's. To circumvent this problem, all further assays to describe the enzyme properties, were performed at pH 7.5 at which 90% of the activity is still left. The incubation time was shortened to 10 minutes.

Influence of divalent metal ions and other reagents

The divalent metal ions Co^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were tested over the concentration range of 1 – 20 mM. One mM of Co^{2+} and Mn^{2+} did stimulate the reaction rate 160 – 180%. With Mg^{2+} a continuous rise in activity was found up to 10 mM; at this concentration there was a two-fold stimulation. EDTA (1 mM, pH 7.5) had hardly any influence upon the reaction rate (95%). The enzyme was for 55% irreversibly inhibited by 1 mM $HgCl_2$, and strongly inhibited by the sulfhydryl specific reagents *p*-chloromercuribenzoate (95%) and *N*-ethylmaleimide (92%). Both cysteine and β-mercaptoethanol could counteract this inhibition only partially.

Molecular weight determination

The molecular weight of the enzyme was determined by the method of Andrews [5] using a Sephadex G-150 column calibrated with cytochrome-*c*, chymotrypsinogen A, egg albumin, aldolase and catalase as standards. The Sephadex G-150 eluate fractions tested showed one maximum corresponding with a molecular weight of 45000 daltons.

Kinetics

A Lineweaver-Burk plot [7] of the reciprocal of the initial velocity of the anthocyanin rhamnosylation reaction *versus* the reciprocal of the UDP-L-

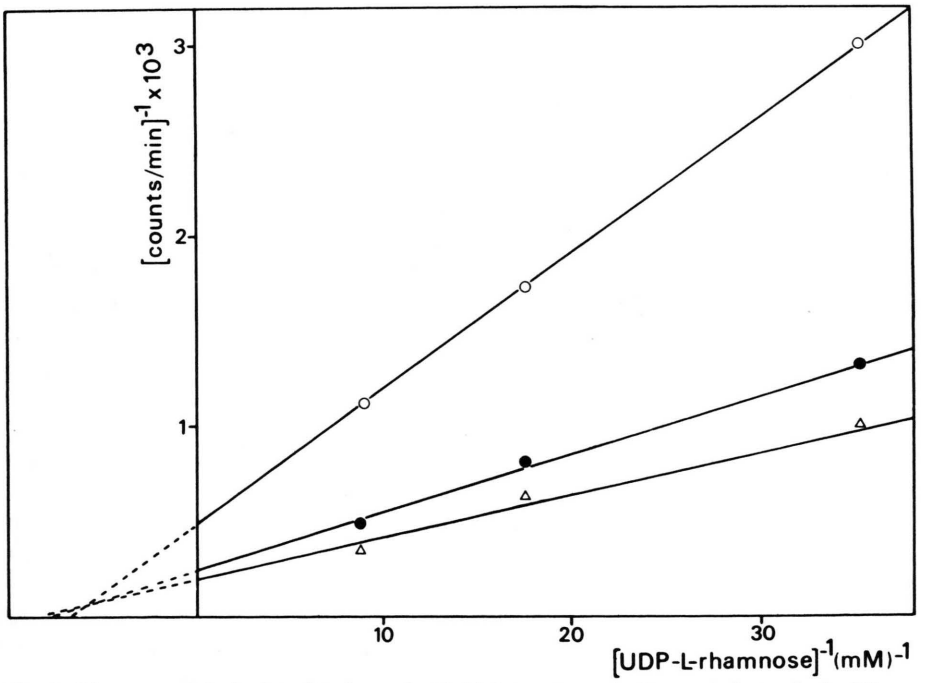

Fig. 1. Lineweaver-Burk plot of reciprocal of initial reaction velocity vs. reciprocal of millimolar concentration of UDP-L-rhamnose. The following final concentrations of cyanidin 3-O-glucoside were used: Δ, 0.83 mM; ●, 0.44 mM; ○, 0.22 mM. The reaction mixture contained in a total volume of 65 µl: 250 nmol potassium-sodium phosphate buffer (pH 7.5), 100 nmol β-mercaptoethanol, 0.01% Triton X-100, 65 nmol $MnCl_2$, 25 µl enzyme, the indicated amount of cyanidin 3-O-glucoside, and UDP-L-rhamnose in a concentration ranging from 0.03 – 0.11 mM.

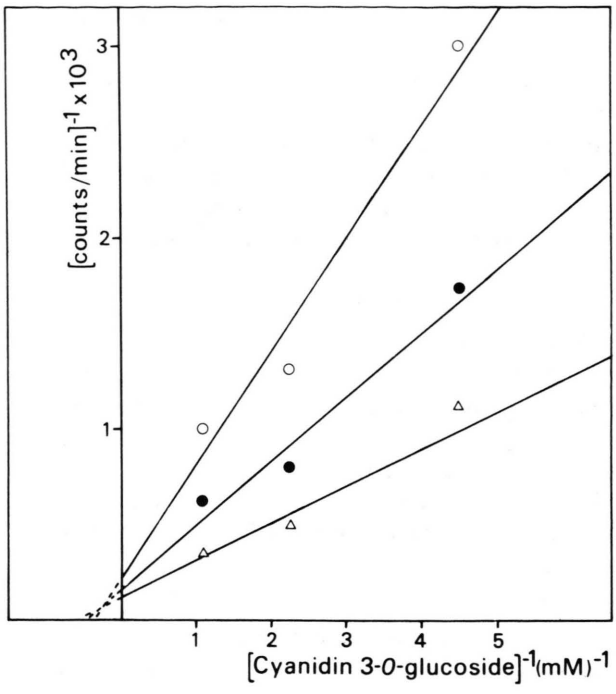

Fig. 2. Lineweaver-Burk plot of reciprocal of initial velocity vs. reciprocal of millimolar concentration of cyanidin 3-O-glucoside. The following concentrations of UDP-L-rhamnose were used: Δ, 0.11 mM; ●, 0.06 mM; ○, 0.03 mM. The reaction mixture contained in a total volume of 65 µl: 250 nmol potassium-sodium phosphate buffer (pH 7.5), 100 nmol β-mercaptoethanol, 0.01% Triton X-100, 65 nmol $MnCl_2$, 25 µl enzyme, the indicated amount of UDP-L-rhamnose, and cyanidin 3-O-glucoside in a concentration ranging from 0.22 – 0.83 mM.

rhamnose concentration at different fixed cyanidin 3-O-glucoside concentrations shows a linear relationship between $1/V$ and 1/UDP-L-rhamnose, the apparent *Km* value being dependent upon the second substrate (cyanidin 3-O-glucoside) concentration (Fig. 1). When the reciprocal of the initial velocity was plotted *versus* the reciprocal of the cyanidin 3-O-glucoside concentration at different fixed UDP-L-rhamnose concentrations, a linear relationship between $1/V$ and 1/cyanidin 3-O-glucoside was observed (Fig. 2). From this figure it follows that the apparent *Km* for cyanidin 3-O-glucoside is also dependent upon the second substrate (UDP-L-rhamnose) concentration.

Secondary plots (Fig. 3), according to Florini and Vestling [8], of the intercepts on the $1/V$ axis *versus* the 1/concentration of the fixed substrate yield the $1/V_{max}$ and the $1/Km$ for each substrate ("true *Km* for cyanidin 3-O-glucoside 2.2 mM, for UDP-L-rhamnose 0.09 mM, and V_{max} 9.5 nmol/min/mg protein).

Substrate specificity

The enzyme was also capable to catalyze the transfer of the rhamnose moiety from UDP-L-rhamnose to the 3-O-glucose of pelargonidin- and delphinidin 3-O-glucoside. The apparent *Km* values of the enzyme for these acceptors are, in the presence of 0.06 mM UDP-L-rhamnose, 1.8 mM for pelargonidin 3-O-glucoside, 2 mM for cyanidin 3-O-glucoside and 1.8 mM for delphinidin 3-O-glucoside.

In the absence of a dominant allele of gene *P* pelargonidin-glycosides are found in petals of *S. dioica*. Up to now no delphinidin-glycosides have been found in petals of *S. dioica*.

The enzyme had a much lower affinity for cyanidin 3,5-diglucoside and pelargonidin 3,5-diglucoside. For the rhamnosylation of cyanidin 3,5-diglucoside a similar pH optimum as mentioned for cyanidin 3-O-glucoside was observed. At this optimum (pH 8.0) the formation of cyanidin 3-rhamnosylglucoside-5-glucoside was less than 20% the formation of cyanidin 3-rhamnosylglucoside (Table III).

Genetic control

The activity of the rhamnosyltransferase in petal extracts of various genotypes of *S. dioica* is presented in Table IV. These data show that the presence of gene *N* is necessary for production of the enzyme that catalyzes the transfer of the rhamnose moiety of UDP-L-rhamnose to the 6-hydroxyl group of the 3-O-bound glucose of anthocyanidin 3-O-glucosides. No 6″-O-rhamnosyltransferase activity was found in petals of *n/n* plants. From this it can be concluded that the anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase exhibits the properties of a rhamnosyltransferase which is controlled by gene *N*.

The enzyme was also present in petals of *p/p m/m N/N* plants which contain pelargonidin 3-rhamnosylglucoside. The rhamnosyltransferase activity in *p/p* plants was as high as in *P/P* plants. This shows that the action of gene *N* is independent of the other anthocyanin modifying genes. The anthocyanidin 3-O-glucosyltransferase [4], which is also present in the extracts of the above mentioned genotypes was not influenced by gene *N*. Both in *N/N* and *n/n* plants this activity could be demonstrated (Table IV).

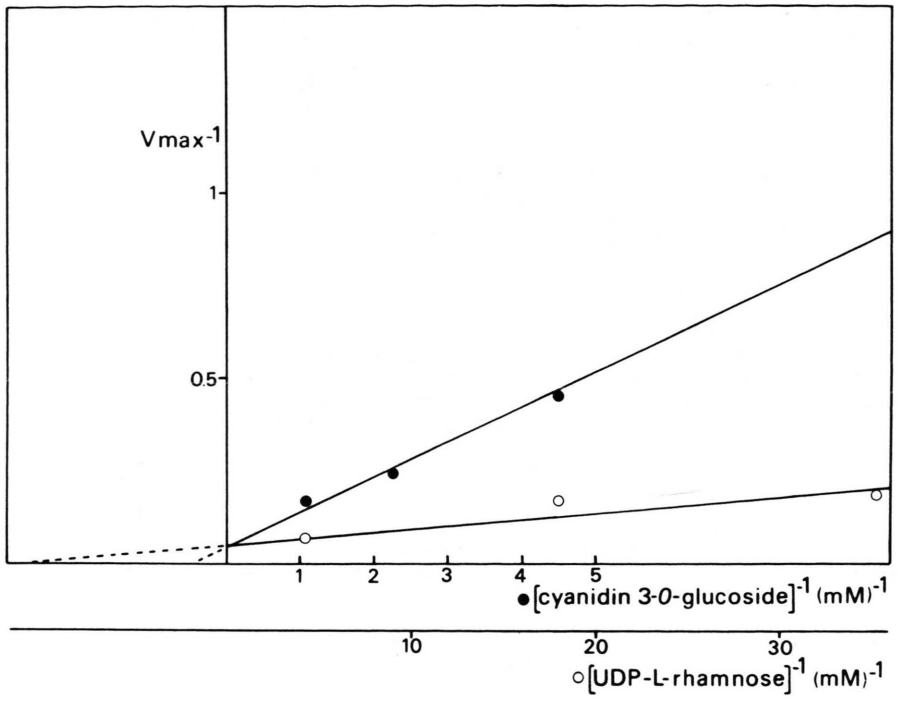

Fig. 3. Plot of the intercepts on the vertical axes of the data plotted in the Fig. 1 and 2 (corresponding to $1/V_{max}$ in the presence of the second substrate as limiting factor) replotted vs. reciprocal of corresponding fixed substrate concentration: ○, 1/UDP-L-rhamnose; ●, 1/cyanidin 3-O-glucoside. The intercept of the vertical axis equals 1/true *Km* UDP-L-rhamnose and 1/true *Km* cyanidin 3-O-glucoside.

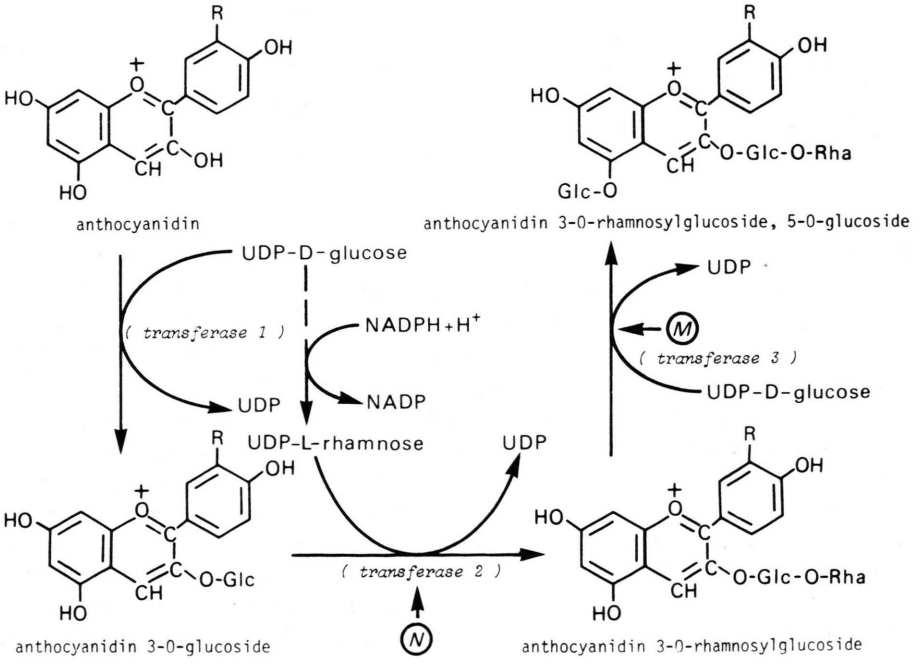

Fig. 4. Biosynthetic pathway of cyanidin- and pelargonidin 3-rhamnosylglucoside-5-glucoside formation in petals of *S. dioica* plants. Transferase 1, UDP-glucose: anthocyanidin 3-O-glucosyltransferase; transferase 2, UDP-rhamnose: anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase; transferase 3, UDP-glucose: anthocyanidin 3-rhamnosylglucoside, 5-O-glucosyltransferase. (R = H, pelargonidin-glycosides; R = OH, cyanidin-glycosides.)

Table III. Substrate specificity of UDP-L-rhamnose: anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase isolated from petals of *S. dioica* plants.

Sugar donor	Acceptor	Carrier	Radioactivity cochromatographed with carrier [cpm]
UDP-L-rhamnose	Cy 3G[a]	Cy 3RG[a]	438
UDP-L-rhamnose	Pg 3G	Pg 3RG	397
UDP-L-rhamnose	Dp3G	Dp3RG	378
UDP-L-rhamnose	Cy 3G 5G	Cy 3RG 5G	87
UDP-D-glucose	Cy 3G	Cy 3RG/Cy 3GG	none
UDP-L-rhamnose	none	Cy 3RG	none

The enzyme was assayed as described in Material and Methods. The assay mixture contained about 100 nmol of anthocyanin acceptor. Radioactivity was determined after cochromatography of the reaction mixture with carrier anthocyanin, on the spot of the carrier and is given as cpm above the background.
[a] Abbreviations: Cy, cyanidin; Pg, pelargonidin; Dp, delphinidin; R, rhamnose; G, glucose.

Table IV. Activity of UDP-L-rhamnose: anthocyanidin 3-O-glucoside: 6″-O-rhamnosyltransferase and anthocyanidin 3-O-glucosyltransferase in leaves and petals of various genotypes of *S. dioica* plants.

Genotype	Anthocyanin present	6″-O-rhamnosyl-transferase activity [cpm/mg protein][b]	3-O-glucosyl-transferase activity [cpm/mg protein][a]
Petals			
C- P- M- N-	Cy 3RG 5G[c]	3303	1350
C- P- mm N-	Cy 3RG	4263	1763
C- P- M- nn	Cy 3G/Cy 3G 5G	none	1378
C- P- mm nn	Cy 3G	none	4054
C-pp mm N-	Pg 3RG	3829	not det.
Green parts			
C- P- mm N-	Cy 3G	none	298

The anthocyanidin 3-O-glucosyltransferase was assayed as described by Kamsteeg *et al.* [4]. [a] cpm incorporated into cyanidin 3-O-glucoside. The 6″-O-rhamnosyltransferase was assayed as described in Material and Methods. [b] cpm incorporated into cyanidin 3-rhamnosylglucoside.
[c] Abbreviations: Cy, cyanidin; Pg, pelargonidin; R, rhamnose; G, glucose.

Discussion

In this paper the rhamnosylation of the 3-O-bound glucose of anthocyanidin 3-O-glucosides is described. It is shown that gene *N* controls the formation of the enzyme, UDP-L-rhamnose: anthocyanidin 3-O-glucoside, 6″-O-rhamnosyltransferase, because no rhamnosyltransferase activity was detectable in petals of *n/n* plants. The enzyme uses UDP-L-rhamnose as rhamnosyl donor. This nucleoside has therefore to be present in petals of *S. dioica.* We demonstrated that UDP-D-glucose is converted to UDP-L-rhamnose both in petals and leaves of *S. dioica* [9]. The enzymes which are involved in this conversion: UDP-D-glucose-4,6-hydrolyase, UDP-4-keto-6-deoxy-D-glucose, 3,5-epimerase and NADPH: UDP-6-deoxy-L-lyxo-4-hexulose, 4-reductase have been demonstrated in petals of both *N/N* and *n/n* plants.

In previous papers [2, 4] two other anthocyanin-glycosyltransferases isolated from petals of *S. dioica* have been described. The anthocyanidin 3-O-glucosyltransferase catalyzes the transfer of glucose moiety from UDP-D-glucose to the 3-hydroxyl group of the anthocyanidin molecule, the anthocyanin 5-O-glucosyltransferase catalyzes the transfer of glucose to the 5-hydroxyl group of anthocyanidin 3-

rhamnosylglucosides. The 5-O-glucosyltransferase is controlled by gene *M*.

In petals of *S. dioica* always cyanidin 3-rhamnosylglucoside-5-glucoside and its acylated form cyanidin 3-(4-caffeoylrhamnosyl(1 → 6)glucoside-5-glucoside are present. The synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside proceeds in a sequential order – first the glucosylation of the 3-hydroxyl group, then the attachment of rhamnose to the 3-O-bound glucose, followed by the glucosylation of the 5-hydroxyl group. The alternative pathway, *i. e.* first glucosylation of the 3-hydroxyl group, then the glucosylation of the 5-hydroxyl group, followed by the addition of rhamnose to the 3-O-bound glucose is less likely as:

1. It was not possible to select for plants with only cyanidin 3,5-diglucoside in the petals. Always a mixture of cyanidin 3-O-glucoside and cyanidin 3,5-diglucoside was found.
2. Anthocyanidin 3-O-glucosides are no appropriate substrates for the 5-O-glucosyltransferase. Although the formation of cyanidin 3,5-diglucoside has been demonstrated to occur at pH 6.5, the reaction at this optimum proceeds 5 – 10 times slower than the 5-O-glucosylation of cyanidin 3-rhamnosylglucosides at pH 7.4 [3].
3. Anthocyanidin 3-O-glucosides are obviously good substrates for the 6″-O-rhamnosyltransferase. This enzyme has a much lower affinity for anthocyanidin 3,5-diglucosides.

The synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside therefore probably occurs according to the pathway as depicted in Fig. 4.

Experimental

Plant material

Silene dioica was grown in the experimental garden of the Department of Population and Evolutionary Biology of Utrecht University. The growing conditions and the crossing methods were the same as published before [11]. The various genotypes were obtained by means of selection and inbreeding [12]. The petals of opening flowers of a given genotype were collected in tubes placed in crushed ice and stored at −20 °C.

Chemicals

UDP-[U-^{14}C]glucose, S. A. 283 Ci/mol, was supplied by the Radiochemical Centre Amersham, England.

Solvent systems

The following solvent systems were used for chromatography: 1% hydrochloric acid (HCl), 15% acetic acid (HOAc), *n*-butanol-acetic acid-water (4:1:5, v/v/v, upper phase) (BAW), acetic acid-hydrochloric acid-water (30:3:10, v/v/v) (Forestal), *n*-butanol-2 N hydrochloric acid (1:1, v/v) (BuHCl), ethyl acetate-formic acid-water (68:14:18, v/v/v) (EFW).

Preparation of substrates

UDP-α-L-[U-^{14}C]rhamnose with a specific activity of 3 Ci/mol was synthesized enzymatically from UDP-D-[U-^{14}C]glucose with a protein preparation of leaves of *Nicotina tabacum* var. Xanthi [10].

Cyanidin 3-O-glucoside and cyanidin 3-rhamnosylglucoside were isolated from petals of *S. dioica* with the appropriate genotype. Pelargonidin 3-O-glucoside was isolated from strawberries [13]. Delphinidin 3-O-glucoside was isolated from *Petunia hybrida.* The anthocyanins were purified by two-dimensional chromatography on Whatman III in the solvent systems BAW and HCl, respectively, eluted with 70% methanol, 0.01% hydrochloric acid and concentrated to dryness under reduced pressure. The anthocyanins were re-dissolved in a small volume of methanol, 0.01% hydrochloric acid, dried over $CaCl_2$ and precipitated in a large volume of diethylether. The precipitate was dried in a desiccator over silica. The anthocyanins used as substrates were dissolved in water/hydrochloric acid, pH 4. The final concentration of the anthocyanin solutions were determined by spectrophotometry (cyanidin 3-O-glucoside: 538 nm (ε 14000), in ethanol, 0.01% hydrochloric acid; pelargonidin 3-O-glucoside: 515 nm (ε 13000) and cyanidin 3-rhamnosylglucoside: 522nm (ε 28200), in ethanol, 0.1% hydrochloric acid [14, 15]).

Protein assays

Protein was determined according to the method of Lowry *et al.* [16] using bovine albumin as a standard. In the presence of interfering substances as PVP, Triton X-100 and β-mercaptoethanol, the calibration curve was also determined in the presence of these substances [17].

Enzyme preparation

All steps were performed between 0 and 4 °C. Five grams of petals were homogenized with an all glas Potter Elvehjem homogenizer in 5 ml 50 mM potassium-sodium phosphate buffer (pH 7.5), containing 20 mM β-mercaptoethanol, 5% soluble polyvinylpyrrolidone with a molecular weight of 44000, and 0.1% Triton X-100, and centrifuged for 15 minutes at $38000 \times g$. To remove endogenous substrates, phenolic compounds, soluble PVP and low molecular materials, the supernatant was passed subsequently through a Polyclar AT (PVP) (1 by 20 cm) and a Sephadex G-50 column (2.5 by 30 cm). Both columns were equilibrated and eluted with 10 mM potassium-sodium phosphate buffer (pH 7.5), containing 4 mM β-mercaptoethanol and 0.02% Triton X-100. Unless otherwise noted the high molecular weight fraction of the Sephadex G-50 eluate which had been concentrated in an Amicon on-line concentrator (CEC 1) with an UM-10 filter (Amicon, Massachusetts), was used as enzyme source.

Assay of enzymatic activity

The standard reaction mixture contained in a total volume of 65 µl, 50 µl enzyme, 146 nmol cyanidin 3-O-glucoside or 138 nmol pelargonidin 3-O-glucoside, 48 nmol UDP-L-[U-^{14}C]rhamnose (S. A. 3 Ci/mol) and 50 nmol $MnCl_2$. The reaction mixture was incubated for 10 minutes at 30 °C, and stopped by the addition of about an equal volume of trichloroacetic acid (10%) in methanol, and transferred quantitatively, together with carrier cyanidin-, respectively pelargonidin 3-rhamnosylglucoside, as a spot on Whatman III paper and developed two-dimensionally in BAW and HCl, respectively. After drying, the anthocyanidin 3-rhamnosylglucoside spot was cut out, placed in a scintillation vial with toluene liquifluor, and counted in a liquid scintillation spectrometer (counting efficiency approximately 80%).

For the determination of zero time control, trichloroacetic acid was added to the incubation mixture before incubation. All enzyme tests were run in duplicate or triplicate.

Characterization of the enzymatically formed product

To determine which part of the anthocyanin formed was labelled, it was suspended in 1 ml 2 N hydrochloric acid and hydrolyzed for 1 h in a fused tube in a boiling water bath [6]. The anthocyanidin formed was extracted with isoamyl alcohol, concentrated under reduced pressure and applied as a spot on Whatman III and developed in Forestal. After drying of the chromatogram, the cyanidin spot was cut out and counted in 20 ml toluene liquifluor in a liquid scintillation spectrometer. The sugar containing water phase was neutralized over a mixed bed ion exchanger (Merck V) and concentrated under reduced pressure. The released sugar was identified by means of cochromatography with glucose and rhamnose on a cellulose MN 300 thin layer plate, developed twice with formic acid- 2-butanone-tertiairy butanol-water (15:30:40:15, v/v/v/v) [18]. The sugar was made visible by spraying with a mixture of 2.58 gram aniline hydrogen phtalate in 100 ml *n*-butanol saturated with water, followed by heating for 5 minutes at 110 °C [19]. The glucose and rhamnose spots were scraped off, bleached by spraying with 30% hydrogen peroxide, and after drying suspended in 20 ml of a instafluor-water mixture and counted.

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